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SOME FACTORS EFFECTING SPERMATOKINESIS IN THE TESTES OF THE HOUSE SPARROW (*PASSER DOMESTICUS*)

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ABSTRACT

This investigation is intended to amplify some of the physical changes at the terminal sequence of spermatogenic activity in the testes of the male house sparrow (*Passer domesticus*): (1) by demonstrating spermatokinesis in excised testes *in vitro* in hypotonic solutions; (2) by demonstrating spermatokinesis in testes *in vivo* following injections of chorionic gonadotropins and epinephrine; and (3) by correlating spermatokinesis and cellular reactions immediately subsequent to hormone injections or *in vitro* treatments of testes with other histologic changes and measurements of testicular volume.

All excised, fixed testes were dehydrated through an ethyl alcohol-xylene series, imbedded in paraffin, sectioned at 5 microns, stained with Harris hemotoxylin and eosin, and mounted in piccolyte. Testes fixed immediately and testes in Ringer's solution did not increase in volume and did not emit sperm. Testes in distilled water increased in volume and emitted sperm.

Following unilateral castration, *in situ* sparrow testes increase in volume after injections of 100 I.U. of A.P.L. or 0.1 mg. of epinephrin.

The histological appearance of sections of variously treated testes correlated directly with volume increase and sperm emission data. Sections from testes fixed immediately or immersed in Ringer's yield a series of cross sections of open, concentrically organized tubules. There is a peripheral-central sequence of progressively more mature spermatogenic stages: spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, maturing sperm clumps and detached, mature sperm in the lumen. Immaturity was recognized in terms of lack of these concentric stages.

Sections from testes immersed in water or from birds injected with A.P.L. and epinephrin reveal partially closed, debris-filled tubules whose concentric organization is disrupted in varying degrees by: (1) expansion and vacuolization of a parabasal zone of primary spermatocytes, (2) extension of Sertoli cells and disintegration of sperm clumps, and (3) sloughing of the voided ends of

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Sertoli cells and the amorphous, achromal masses which lie between sperm clumps. There is no significant variation of intertubular spaces or tissues in any of the preparations discussed in this study.

PRESUMABLY because frogs and toads are extensively used in mammalian pregnancy tests and for other laboratory and teaching purposes, their hormonally controlled processes of spermatogenesis and sperm release have been studied thoroughly. It is, therefore, not surprising that the first causal explanations of spermatokinesis are in reference to this group of vertebrates. Rugh (1) suggested that the contractile force of smooth muscle cells in the capsular and interstitial tissues of the grassfrog testis supplies sufficient pressure to force sperm out of the seminiferous tubules. McCourt (2), as well as others who have sought to verify this condition reported no positive evidence of the presence of smooth muscle in the testes of the grassfrog. He reported, however, an anterior-posterior gradient of sperm maturation and release in the testes of frogs repeatedly stimulated by injections of chorionic gonadotropic hormones.

The investigations of DeRobertis (3), van Oordt, *et al.* (4) and Giltz (5) indicate that a hydrostatic pressure presumably resulting from fluid accumulations in Sertoli cells initiates sperm release when these cells rupture. Moreover, the reports of these and other investigators take note of the consistent fact that testes enlarge and are heavier immediately prior to sperm release under various experimental conditions.

There is apparently no available account in the literature explaining the mechanism of spermatokinesis within the seminiferous tubules of the testes of an amniote vertebrate. Nelsen (6) suggests that fluid pressure forces sperm out of the testes of all vertebrates, including birds; however, he cites no source of information. The proximity of the vascular elements of the metanephric kidney and the testes in these higher vertebrates is less pronounced than in the mesonephric kidney of the frog, toad, and other anamniote vertebrates. Hydrostatic pressure changes might be demonstrated more easily in closely connecting gonadal and renal systems, as occurs in the frog and toad, than in distinctly separate systems as occurs in the reptiles, birds, and mammals. It is the purpose of this investigation to explain in part some of the physiological and morphological changes coincident with, and presumably operative in, the release of sperm within the tubules of the testes of the house sparrow.

MATERIALS AND METHODS

The sparrows used in this study were trapped locally. The gonadotropic hormones used were Ayerst brand of human chorionic gonadotropin (A.P.L.). The epinephrin used was Parke, Davis and Company adrenalin hydrochloride.

Testes of mature birds removed for *in vitro* studies were immediately measured using a screw-adjustment divider and a millimeter ruler. For each testis the long axis and the diametrical axes perpendicular to each other were determined and recorded at the beginning and at the end of specified periods of experimental treatment as described below.

With these measurements the volumes of the testes were calculated as cubic millimeter volumes of oblate spheroids by the formula $V = 4/3\pi a^2 b$, where a is one-half the long axis in mm. and b is one-half the average of the two diametrical axes in mm. The difference between the initial and final volume was calculated and the percentage change in volume was determined to permit better correlation of volume change with sperm release or histologic change. The testes from the same bird were compared in all cases in their reactions to fluids of different osmotic tonicity. All fluids were at approximately 25° C. This procedure provided three kinds of optimal control-comparison situations. The first involved immediate fixing of one member of a pair of testes, while the other testis was immersed in distilled water for a specified period of time before fixing. The second comparison utilized the same procedure except that the second member of each pair of testes was immersed in Ringer's solution for warm-blooded animals. The third comparison was effected by simultaneous immersion of one testis in Ringer's solution and the other in distilled water for an equal length of time before fixing.

The testes were measured after the period of experimental treatment and then fixed 18–24 hours in Zenker's fluid and washed 18–24 hours in running tap water. These specimens were dehydrated and cleared through an ethyl alcohol-xylene series and imbedded in paraffin. Sagittal sections at 5μ were stained with Harris' hematoxylin and eosin and mounted in piccolyte. From each testis a representative section was described on the basis of 3 major criteria; spermatogenic activity, organization of seminiferous tubule cross sections and intertubular histology.

Comparison between members of each pair of testes during the *in vivo* phase of the study was accomplished by first removing the left testis from a bird anesthetized with sodium pentobarbital (Nembutal, Abbott). One hundred I.U. of A.P.L. or 0.1 mg. of epinephrin was injected intramuscularly and the remaining testis was excised after 30, 60, 120, 240, and 480 minute intervals. After the first member of the pair of testes was excised, both were measured. Because the *in situ* testis is rather inaccessible, accurate meaurement was not always certain. The most accurate measurement obtainable gave a fair approximation of "pre-treatment" dimensions of these testes. This method made possible calculation of control or "pre-treatment" volumes of the treated testes. These testes were also fixed in Zenker's fluid and prepared for sectioning in the manner previously described. A representative section from each slide was described with reference to spermatogenic activity, organization of seminiferous tubule cross-sections, and the intertubular histology. The histological appearances of the testicular sections were correlated with gross and volumetric changes noted during the experimental treatment.

RESULTS

Comparison between immediately fixed and water-immersed testes showed an appreciable increase in the volume of the water-immersed testis (Table 1). Sperm were released into the water after one hour, if the testis was mature. Also conspicuous changes were noted in the morphology of the seminiferous tubules. This was not the case with Ringer's-immersed testes. In these there was little or no volume change, and likewise no significant histologic change when compared to immediately-fixed testes.

As sparrow testes recrudesce, the germinal epithelium proliferates at such a rapid rate peripherally in the seminiferous tubules that the central cavity or lumen forms in a manner which suggests the development of an elongate blastocoel of a vertebrate embryo. The sexually mature testis presents a mass of enlarged tubules whose lumens are conspicuously lined with

TABLE 1. VOLUME RELATIONSHIPS IN SPARROW TESTES. THE DIMENSIONS AND VOLUMES OF SPARROW TESTES WITH REFERENCE TO FIXING IMMEDIATELY, IMMERSION IN RINGER'S SOLUTION AND IN WATER, AND TO HORMONE INJECTIONS

Treatment	Number of testes	Average volume before treatment	Average volume after treatment	Average % change in volume	Sperm in fluids
Fix immediately	85	261 mm.³			neg.
Ringer's solution					
30 min.	10	235 mm.³	241 mm.³	2.2%	neg.
60 min.	5	293 mm.³	302 mm.³	3.3%	neg.
120 min.	15	224 mm.³	226 mm.³	0.9%	neg.
240 min.	16	266 mm.³	269 mm.³	1.5%	neg.
480 min.	2	286 mm.³	286 mm.³	0.0%	neg.
Distilled water					
30 min.	25	251 mm.³	303 mm.³	22.2%	neg.
60 min.	10	281 mm.³	352 mm.³	28.1%	pos.
120 min.	30	297 mm.³	370 mm.³	27.3%	pos.
240 min.	15	300 mm.³	407 mm.³	35.9%	pos.
480 min.	3	306 mm.³	386 mm.³	33.3%	pos.
100 I.U. A.P.L. intramuscularly					
60 min.	15	309 mm.³	402 mm.³	31.0%	Testes *in situ*
120 min.	15	283 mm.³	367 mm.³	30.0%	
240 min.	6	300 mm.³	386 mm.³	31.1%	
300 I.U. A.P.L. intramuscularly					
120 min.	10	275 mm.³	361 mm.³	31.6%	
240 min.	15	272 mm.³	366 mm.³	36.5%	
0.1 mg. epinephrin intramuscularly					
120 min.	8	291 mm.³	330 mm.³	18.0%	
240 min.	12	299 mm.³	366 mm.³	22.0%	

meiotic figures. Interstitial tissue is relatively less abundant because it does not proliferate as does the intra-tubular tissue. The concentric organization of the mature tubule is a peripheral-central sequence of spermatogenic cell types from the inactive spermatogonia through the fully metamorphosed, detached sperm. Attention was focused on the spoke-like arrangement of dark-staining, maturing sperm with heads buried in the ends of the Sertoli cells. Although there is considerable variation in the whole of any testis, the sperm clumps are usually compact and aligned along the margin of the zone of primary spermatocytes. Between and central to the clumps of sperm and their tails, which in fully mature tubules have a wavy appearance, lies an acellular, achromal mass of cell debris, sloughed cytoplasm from metamorphosing spermatids, and degenerating terminal portions of Sertoli cells. Variations of any consequence in the intertubular spaces and interstitial tissues were not observed in histological comparisons of the testes of variously treated birds.

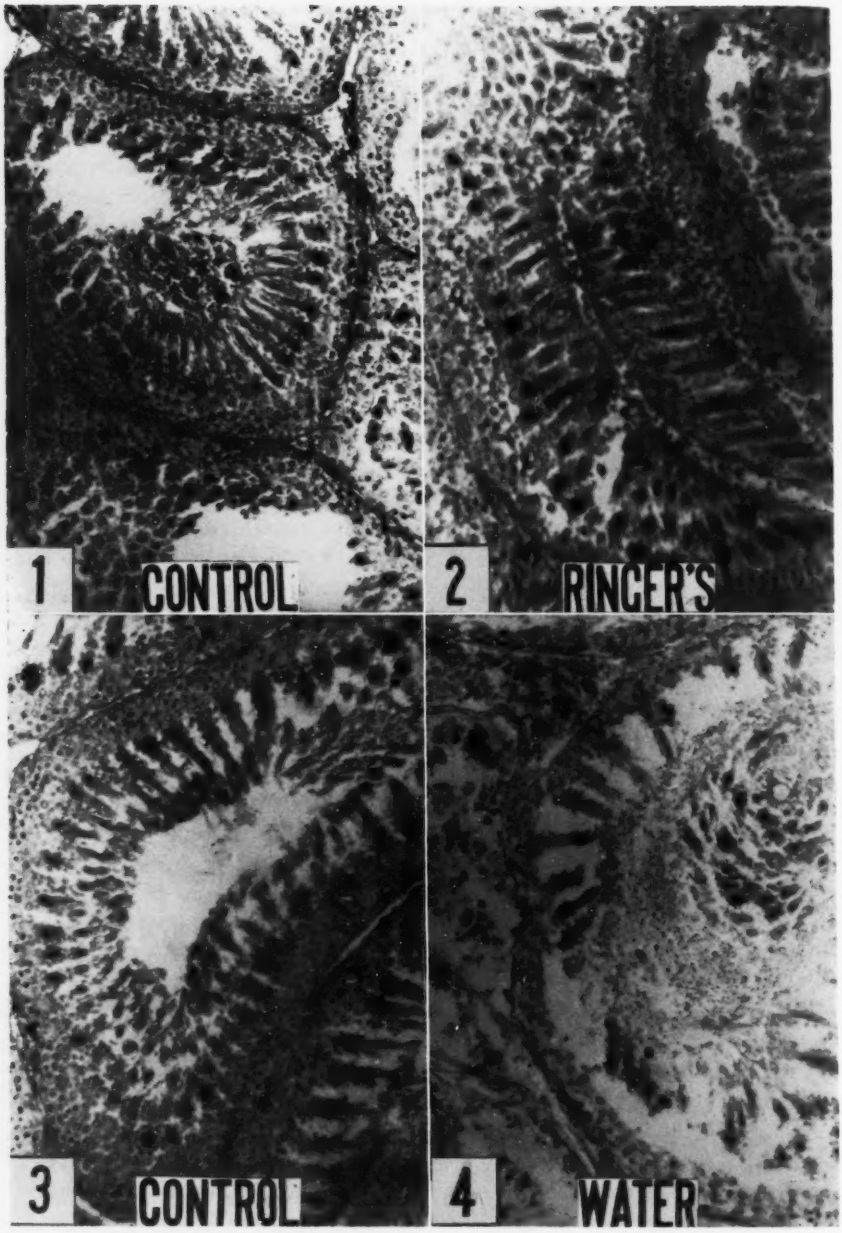
The histologic appearance of Ringer's-immersed testes do not differ significantly from those fixed immediately, except for necrotic changes beginning usually after 1–2 hours. (cf. Fig. 1 and Fig. 2).

The degree of volume increase of water-immersed testes appears to be a function of time, within the limits of 3–4 hours (Table 1). When the volume of mature sparrow testes in water increases approximately 30%, sperm are usually observed in the water. This critical volume is usually achieved between one-half and one hour, and constitutes most of the volume change. There is usually a gradual increase in volume, after the critical volume is reached, to a maximum volume in 3–4 hours. Whenever a testis increased in volume beyond the critical volume and sperm were not seen in the water, the testis almost always proved to be immature. Conversely, when sperm appeared in Ringer's solution or especially soon in water, damage to the tunica albuginea usually was noted macroscopically or microscopically.

The histological appearance of sagittal sections of water-immersed testes is strikingly different from those fixed immediately and those immersed in Ringer's prior to fixation. (cf. Fig. 3 and Fig. 4). The concentric organization of the normal tubule cross-section is disrupted primarily between the basement membrane and the ring of sperm-head clumps during the first hour in water. The primary spermatocytes here expand conspicuously and after an hour usually are ruptured, thereby sloughing into the tubular cavity sperm clumps and germ cells in various stages of meiosis. In the initial stages of water immersion the tubular elements between the clumps of sperm erode and along with sloughed cytoplasm from metamorphosing spermatids and ruptured Sertoli cells constitute a mass more dense in the tubule cavity than in those testes fixed immediately or immersed in Ringer's solution. Within four hours the walls of many tubules in these water-immersed testes are reduced to low bands of spermatogonia, a few early phases of primary spermatocytes and basal parts of Sertoli cells.

When A.P.L. is injected into a sparrow the gross changes which occur vary with time from the initial activity of the hormone. There is a rapid initial increase in the volume of the testis, followed by a slight increase after two hours (Table 1).

Mature testes removed a few minutes after the administration of 100 I.U. of A.P.L. are not different from control testes as seen in sagittal sections. The appearance is generally like that described for the Ringer's-immersed and immediately-fixed testes discussed previously. By the end of the first hour after A.P.L. injections, the ring of meiotic cells and the disorganized achromal, acellular debris between the sperm clumps become slightly dislodged. These elements appear to move into the tubular cavity more uniformly than their counterparts located in the control testis (cf. Fig. 5 and Fig. 6). There is no consistent evidence that the distended area ruptures as a sheet of cells and sloughs off as in water-immersed testes, but the degree of expansion is quite noticeable. The erosion of meiotic cells and achromal elements from between the sperm clumps is equally apparent by this time. In fully mature tubules the sperm clumps begin to break apart, discharging mature sperm. Absolute counts were not made, but whole



EXPLANATION OF THE FIGURES

All tissues were fixed in Zenker's fluid, section at 5μ and stained with Harris' hemotoxylin and eosin.

areas of voided Sertoli cells were noted. In contrast, completely intact sperm clumps, often in the cross-section of the same tubule, were seen. Between these extremes of organization of sperm clumps all gradations of sperm dislodgement were observed. The completely voided areas leave a residue of amorphous material that stains approximately as intense as the cytoplasm of other cells. Usually imbedded in this achromal mass is a longitudinally oriented mass of wavy striations which may correspond with the fibrils described by Maximow (7). In the water-immersed testes sperm are released from Sertoli cells first individually as in A.P.L.-injected testes, but soon the sperm clumps dislodge and disintegrate in the lumen of the tubule.

Judged by measurements of the remaining testis *in situ* after unilateral castration in sparrows the gross changes in the testes of epinephrin-injected sparrows are similar to those of water-immersed testes (Table 1).

The initial spermatokinetic effects of epinephrin on the tubules of mature sparrow testes are perhaps more rapid than those produced by A.P.L. The zone of meiotic cells expands slightly and becomes vacuolated. The amorphous, achromal mass of cytoplasmic debris forms a concentric band central to the ring of sperm clumps and tails, but usually the lumen is empty. At the end of four hours the sloughing process is well advanced, the vacuolized band of primary spermatocytes in many areas ruptures in the same zone as that of the epithelium of water-immersed, mature testes (cf. Fig. 7 and Fig. 8). The loss of compactness of sperm clumps is evident in

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FIGS. 1 and 2 are of testes from the same sparrow.

FIG. 1. Section through the testis of a sexually mature sparrow which was fixed immediately after removal. Note the regular, concentric arrangement of the seminiferous tubule, the accumulation of amorphous debris and sperm, and the uniform sloughing of mature, detached sperm. (300×).

FIG. 2. Section through the other testis from the same sparrow fixed after four hours of immersion in Ringer's solution for warm-blooded animals. (300×). The essential organization is judged to be about the same as that of the tubule above, but necrotic changes are so extreme that descriptions are not certain. Note that the first sign of breakdown of the tubule wall is in the area of primary spermatocytes between the ring of sperm clumps and the basal layers of spermatogonia. (300×).

FIGS. 3 and 4 are of testes from the same sparrow.

FIG. 3. Section through testis of a sexually mature sparrow fixed immediately after removal. (300×).

FIG. 4. Section through the other testis from the same sparrow fixed after four hours of immersion in distilled water. Note the extensive breakdown of the tubule wall especially at the zone between the ring of sperm clumps and the basal layers of spermatogonia and epithelial cells, the numerous detached sperm and amorphous debris in the lumen. Part of this change is thought to be due to absorption and part due to necrosis. (300×).

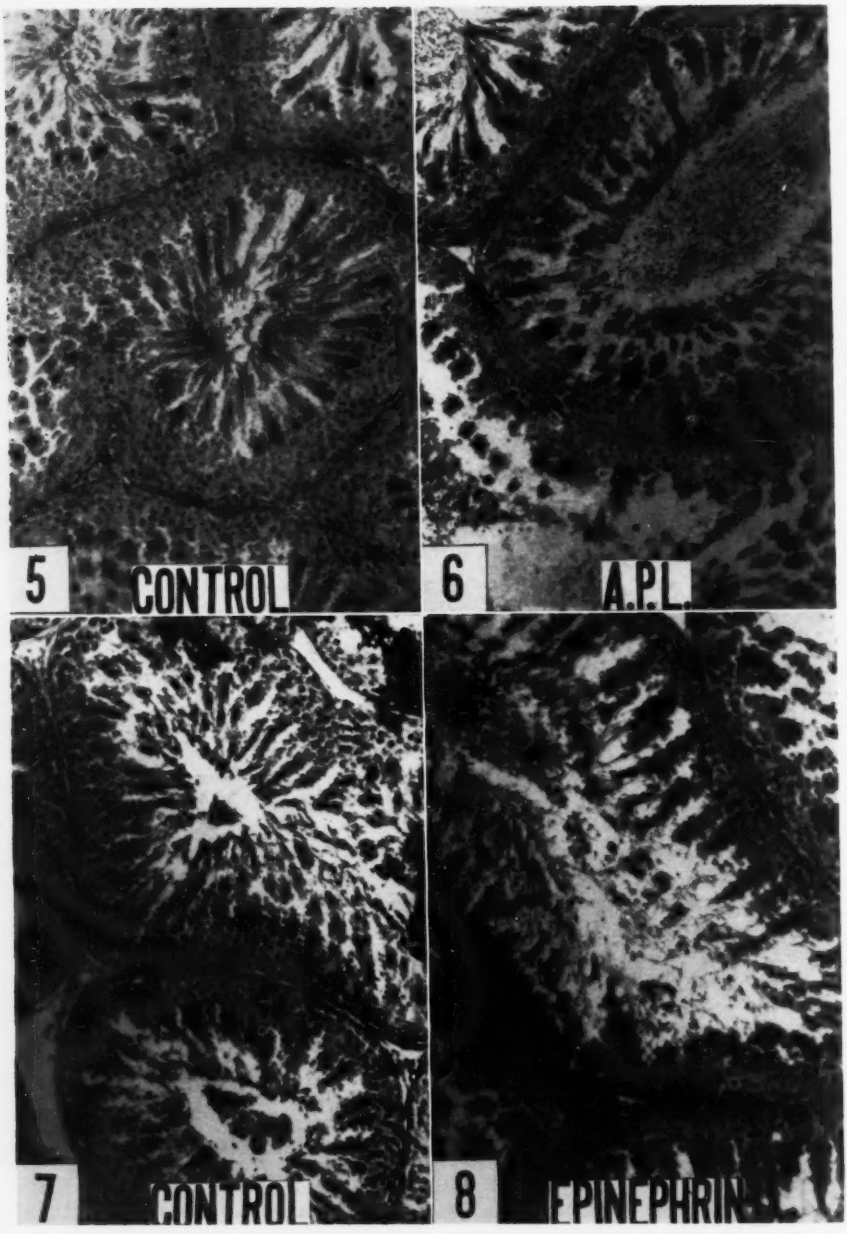

FIG. 5. Section through the testis of a sexually mature sparrow fixed immediately after castration. (300×).

FIG. 6. Section through the other testis of the same sparrow castrated and fixed four

almost all tubules as indicated by the abundance of detached sperm in the lumen. In a few cases whole sperm clumps are sloughed intact and can be seen in various stages of decomposition within the tubule cavity. The erosion between sperm clumps is extensive in all areas and leaves some tubules reduced to the basal elements. Where sections were taken after the luminal mass had passed, the tubule was widely open and bordered only by basal epithelium and spermatogonia.

DISCUSSION

The development of the concept of how gonadotropic hormones and epinephrin function in the testicular and vasa deferens release of sperm began to take form when Rugh's (1) description of smooth muscle in the stroma of the testes of the grassfrog proved inaccurate. However, his earlier correlation of frog pituitary injections with sperm release has been adequately verified many times, and again in this study (Fig. 6). Giltz (5) in attempting to explain how excessive water absorption from frog lymph sacs causes sperm release found he could reproduce the same phenomenon by immersing the excised testis in hypotonic solutions. When similar results were obtained from sparrow testes immersed in water, but were not obtained from testes immersed in Ringer's solution, it appeared that a physical force produced by the diffusion of water into the cells was acting. (cf. Fig. 2 and Fig. 4). The histological changes which water produces in excised testes include swelling and distention of tubular elements, especially primary spermatocytes, and liberation of metamorphosed sperm from clumps. This physical force of water distention of tissue could occur in the intact bird and account for a sufficient source of fluid pressure.

The changes which occur in testes *in situ* are very similar to those described as taking place in water-immersed testes. If this is the case perhaps the same physical forces associated with water absorption into cells operates in the intact testis after an injection of A.P.L. or epinephrin. The same

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hours after the injection of A.P.L. intramuscularly. Note the extensive sloughing of cellular debris from the area between the sperm clumps and also the numerous detached, mature sperm in the lumen. (300×).

FIGS. 7 and 8 are of testes from the same sparrow.

FIG. 7. Section through the testis of a sexually mature sparrow fixed immediately after castration. (300×).

FIG. 8. Section through the other testis from the same sparrow castrated and fixed four hours after the injection of epinephrin intramuscularly. Note the sloughing of tubule elements especially between the sperm clumps. Note also the accumulation of numerous detached, mature sperm in the lumen along with much of the amorphous, acellular debris. The absence of this epithelial debris and many of the sperm clumps give the appearance of a more extensive lumen. This is more typically the result in a tubule where most of the sperm are completely metamorphosed. (300×).

or similar histological changes accompany spermatokinesis in mature sparrows but there is no evidence that precisely the same mechanisms are operating. (cf. Figs. 6 and 8) DeRobertis, *et al.* (3) describe in the toad the expansion and rupture of Sertoli cells which liberate sperm into the lumen of seminiferous tubules in response to implants of toad anterior pituitary. Van Oordt *et al.* (4) describe a similar sequence of events in the release of mature sperm in the testes of the grassfrog. These findings seem to support the conclusion that in the sparrows used in this study water absorption of a sufficient degree causes the rupture of Sertoli cells and the subsequent release of sperm. This phenomenon is perhaps effected by hormone induced general increase in permeability of cell membranes to water. Changes associated with meiotic cell division are known to include alterations in the viscosity of the cytoplasm. Changes in membrane polarization of dividing cells also indicate an increase in the absorption of water.

The similarity of the response to A.P.L. and epinephrin does not necessarily indicate that the mechanism is the same in each case or that there is even a direct action by A.P.L. and epinephrin on the membranes of the cells discussed. While this study is an attempt to ascribe a physical response to A.P.L. and epinephrin at the cellular level, the description is still morphological in essence and provides little insight to the chemical nature of the reactions involved.

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ADVERSE EFFECTS OF CROWDING ON LACTATION AND REPRODUCTION OF MICE AND TWO GENERATIONS OF THEIR PROGENY[1]

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ABSTRACT

Each of 7 populations of 20 male and 20 female mature mice (isolated since weaning) was placed in a 13 in.×18 in. cage for 6 weeks which constituted experimental crowding. No young were born during this period. The mice were established as pairs after crowding, one pair per cage. High mortality of males during crowding restricted the number of pairs available to 88, of which 68 had litters and 56 pairs weaned litters after segregation into pairs. Weights of progeny were obtained at birth and each of the 56 litters was matched and half litters switched with one of equal size and age born to a female which had remained isolated. Weights of the young were obtained weekly for three weeks. The mean weights at birth of progeny of previously crowded and of isolated mice did not differ. Young nursed by crowded females were 15% lighter at weaning than those nursed by isolated mice ($P < 0.001$). The weights of progeny did not differ significantly with respect to which mother bore them or to whether they were nursed by foster or by their own mothers. The mean weight of progeny nursed by crowded or by isolated mothers decreased with increasing litter size. Progeny from litters of 6 or more nursed by crowded mothers weighed significantly less (17%) than those nursed by isolated females, but the weights did not differ significantly for litters of 5 or less.

Young nurtured by crowded mice were paired and their litters similarly matched and half litters exchanged with ones of similar age and size born to isolated mothers. Progeny nurtured by females in turn nurtured by crowded mothers weighed 8% less than their controls ($P < 0.06$). They averaged 18% lighter than their controls for litters of 8, 9 and 10 ($P < 0.005$) but were not significantly lighter in smaller litters.

It was concluded that suppressed growth of progeny nurtured by crowded mothers, persisting for at least 2 generations, was due to quantitatively and/or qualitatively deficient lactation resulting from crowding. Such attenuation of the effects of crowding may explain the long-continued decline in natural populations following peak levels and a precipitous crash in numbers.

An eighth population of 10 male and 10 female mice was sacrificed after 6 weeks of crowding and compared with 10 littermate segregated pairs for reproductive function. Uterine implantation scars were present in all females of both groups, but only 3 of the crowded females bore litters or ever appeared grossly pregnant, whereas all of the segregated females bore litters. The number of implantations per mouse and number of young per litter were significantly

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[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

less in the crowded females. It was concluded that crowding resulted in intra-uterine mortality and probably diminished fertility, explaining why none of the mice in the preceding 7 populations ever appeared pregnant during crowding and why almost half never bore litters, even after segregation.

THE importance of reproduction to the growth and survival of a population is in its ability to produce reproductively competent individuals (1). A decline or failure in the productivity[2] of a population therefore may result from (a) decreased fertility,[2] (b) failure of the zygotes to implant properly, (c) increased postimplantational intra-uterine mortality, (d) post-parturient failure to survive, or (e) failure of the young to mature properly. Deficient lactation could produce mortality of the nursing young directly by starvation or indirectly by inanition with a resultant decreased resistance to disease or environmental exigencies. The developmental stage at which reproductive failure occurs is unimportant in terms of the immediate population, but is important if the adverse effects are such that the progeny are permitted to survive with diminished reproductive competency.

Decreased productivity in mammalian populations associated with increased population density has been demonstrated with house mice and voles in the laboratory with voles and Norway rats under natural conditions (2–13). Data collected from natural populations of other species suggest a similar relationship (14, 15, 16). Investigators agree that decreased productivity is associated with a cessation of population growth, but there is no general agreement on the causes for the diminished productivity. Several mechanisms have been implicated and any or all of them could participate in reducing productivity coincident with increasing population density. A decline in the activity of the reproductive organs occurs in response to increased population density (7, 10, 11, 12). House mice from populations of high density exhibit increased prenatal mortality (12). Increased mortality of young prior to and immediately following weaning has been associated with increased density in several studies (4, 5, 6, 8, 9, 12, 17). Mortality during nursing may be due to altered maternal behavior (6, 9) or to deficient lactation (12, 18). Young house mice and red-backed voles from populations of high density either fail to mature or their maturation is significantly delayed (12, 13). Thus increased mortality of the young associated with increased population density occurs at all stages of fetal and postnatal development. It is possible that all of these forms of mortality are attributable to a common mechanism: The physiological

[2] Productivity is defined for the present purposes as the production of reproductively competent recruits for a population. Fertility is used in a restrictive sense meaning the ability to produce viable ova and sperm capable of producing viable zygotes. Fecundity is defined as the ability to give birth to living progeny. Productivity obviously includes fertility, fecundity and lactational competency.

responses to intraspecific competition and its sequelae (10, 11, 12, 19, 20, 21, 22, 23).

Intra-uterine or postparturient factors adversely affecting the ability of surviving progeny to reproduce would seriously affect the future of their populations. Inadequate nutrition during nursing or intra-uterine physiological derangements conceivably could produce such manifestations. Chitty (17) observed that the progeny of voles from dense populations were more susceptible to disease and less resistant to various stressors. He first suggested that such diminished resistance might result from intra-uterine physiological derangements but later proposed that inadequate maternal lactation probably accounted for the changes in the progeny. His proposal was supported by limited experiments with voles in which he was able to demonstrate diminished growth in the progeny nursed by previously crowded mothers (18). He was unable to demonstrate any prenatal effects. Christian (12) suggested that there was a suppression of lactation in house mice from dense populations since no change in maternal behavior was observed and the stomachs of nursing young were devoid of milk. The progeny in these experiments were stunted, weaned early, and most were unable to survive the immediate postweaning period. Furthermore the survival rate of nursing progeny was inversely related to the logarithm of the number of mice in the population.

Increased population density produces adrenocortical hypertrophy, decreased activity of the reproductive organs, decreased reproductive performance, and other endocrine changes usually associated with pituitary-adrenocortical stimulation (10, 11, 12, 23). The magnitude of these changes is related to the logarithm of the population size (12, 23). It was logical to suppose that lactation would reflect the altered endocrine functions brought about by increased population density, as a variety of stressful stimuli may diminish lactation (24).

The following experiments were designed to show (a) whether crowding female mice results in a subsequent lactational deficiency, and if so (b) how it affects subsequent generations, and (c) to determine at what developmental stages reproductive losses may occur in mice as a result of crowding.

METHODS[3] AND PROCEDURE

Effects of previous crowding on lactation

A standard procedure (diagrammed in Fig. 1) was used for all experimental populations. Twenty male and 20 virgin female albino mice of the NMRI strain were taken at maturity (about four weeks after weaning) and placed in a 13″×18″×7″ cage for six weeks. Food in excess of usage was distributed over the bottom of the cages and water was available from several sources to avoid competition for these items. This pro-

[3] The authors are indebted to Mr. James S. Reid and Robert Tenney, HN, for technical assistance and to Mr. William Cochran for statistical advice.

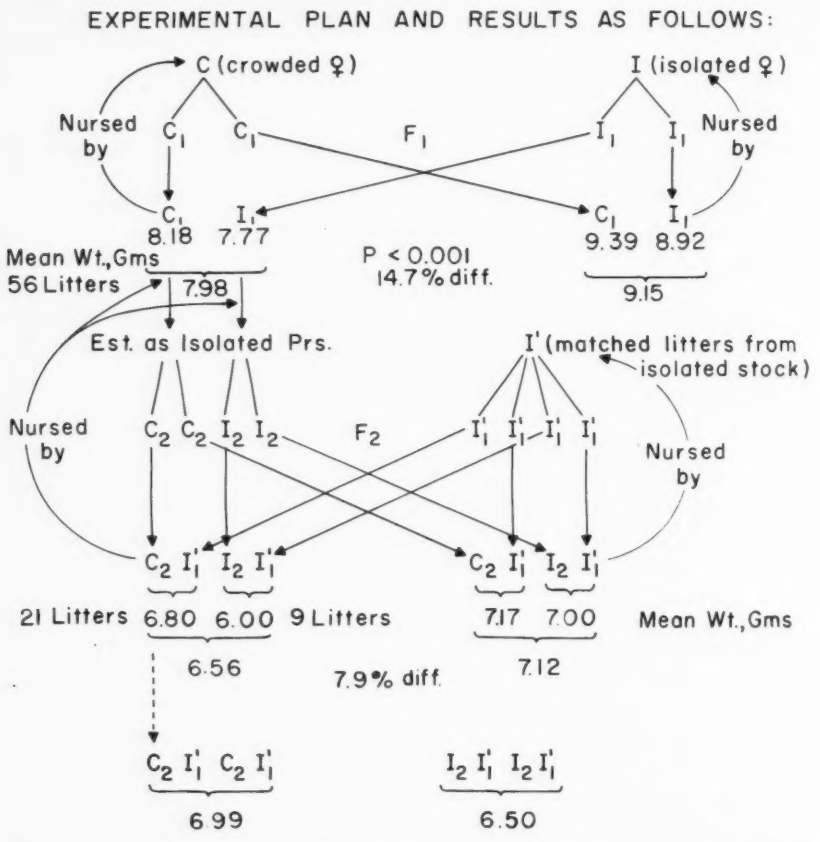

FIG. 1. Effects of maternal crowding on lactation. Seven populations of 20 pairs of mice each crowded for six weeks, then established as single pairs. Mean weight per half litter used as the unit of measurement.

cedure constituted experimentally crowding the mice. Seven such populations were used (Table 1). None of the females in these populations appeared grossly pregnant nor were any litters born during the period of crowding. In no population did all of the males survive crowding. Females occasionally succumbed but the number dying was much smaller than for the males. It has been shown previously that deaths can rarely be attributed to fighting *per se* (10–12), and in any event fighting essentially ceases after the first day or two of crowding.

The mice surviving six weeks of crowding were segregated in pairs, and each pair put in a cage of its own. These segregated pairs were checked daily for litters. When a litter was born, it was weighed *in toto*, the number of young counted and each one marked by toe clipping. Half of this litter was then exchanged with half of one of the same size, age, and parity belonging to a mother which had been segregated since weaning with her mate. Therefore a previously crowded female nursed half of her own and half of a litter born by an isolated mother, and vice versa. The pups were then individually

TABLE 1. SUMMARY OF THE SURVIVAL AND PRODUCTIVITY OF MICE CROWDED 20 PAIRS TO A CAGE FOR 6 WEEKS

Note that the data for three populations were combined for experiment 2. The variation in the number of pairs surviving crowding is due to the differences between populations in the numbers of males which survived the 6 weeks of crowding. See the text for a discussion of this variation in the mortality of males. Note also the general agreement between the percentages surviving and the percentages of the surviving pairs which had litters.

Expt.	No. pairs crowded	No. pairs after 6 wks. crowding	% pairs surviving crowding	No. pairs having litters	% surviving prs. having litters	No. litters weaned	% litters weaned	Mean pup wt.		Wt. of pups crowded as % of isolated
								Raised by crowded mother	Raised by isolated mother	
1	20	13	*65*	7	*54*	5	*71*	6.20	8.08	*77*
2	60 (3×20)	44	*73*	36	*82*	29	*81*	7.79	8.95	*87*
3	20	6	*30*	4	*67*	2	*50*	7.30	8.48	*86*
4	20	8	*40*	5	*63*	4	*80*	8.38	8.82	*95*
5	20	17	*85*	16	*94*	16	*100*	8.59	9.94	*86*
Total	140	88		68		56		38.26	44.27	
Mean (per pop.)	20	12.6	*63*	9.4	*77*	8.0	*86*	7.65	8.85	*86.4*

weighed weekly for three weeks. The mean weight per pup was determined each time for the four half-litters: (1) born and nursed by crowded females, (2) born and nursed by isolated females, (3) born to crowded and nursed by isolated females, and (4) born to isolated and nursed by crowded females (see Fig. 1). The mean weight per infant per half-litter was the unit of measurement, but the 56 degrees of freedom available for statistical analyses were determined by the number of matched whole litters. The effect of crowding the mothers on the weights of the infants at birth was determined for 51 of the 56 pairs of litters.

Effects of crowding on lactation in the second generation

Progeny nursed by crowded females were segregated in pairs at weaning and the entire procedure repeated for another generation, except that none was ever crowded to determine the effects on the F_2 generation. The litters of the progeny were pair-matched with litters of isolated females and half-litters switched between the two mothers, one experimental and one control. The procedure is outlined in Figure 1, but it was carried to the F_2 generation for only the first four populations and, with inevitable losses in the progress of the experiment, only 30 matched pairs of litters from the F_2 generation were available for measurement.

Effects of crowding on reproduction

A progressive loss of productivity with each stage of the reproductive process was suggested by the data in Table 1. There appeared to be a relationship between the number of pairs surviving crowding and the number having litters. The failure of the females to reproduce during crowding did not appear to be due to inadequate male fertility, as twenty-four of the fifty litters from the first four experiments were born between three and four weeks following removal from the crowded conditions (Table 2). This indicates

TABLE 2. DISTRIBUTION OF LITTERS BORN FOLLOWING CROWDING FOR THE FIRST FOUR EXPERIMENTS WITH RESPECT TO THE NUMBER OF DAYS AFTER REMOVAL FROM CROWDED CONDITIONS

	Days after removal from crowded cages											
	21	22	23	24	25	26	27	28	29–35	36–42	43–49	50–67
Number of litters born	1	1	8	0	4	5	3	2	11	2	7	5

that the males had mature sperm and were capable of fertilizing the females during the first estrus following crowding. Furthermore, these males were distributed so that each crowded population contained at least one of them. These results, coupled with an absence of grossly apparent pregnancies during crowding, raised the question of whether decreased fertility, increased intra-uterine mortality or both were responsible for the reproductive failure. If the females became pregnant during crowding, but lost their young *in utero*, deficient male fertility is automatically ruled out as a contributing cause. The following experiment was designed to explore the above questions.

Forty mice, 20 littermate pairs of the same age, were individually caged at weaning. Six weeks later, each of 10 pairs was segregated, one pair to a cage. The remaining mice were put in a single cage, which constituted experimental crowding. Littermate pairs were divided equally between the crowded and segregated groups. The mice were checked daily for litters or for grossly apparent pregnancy in the case of the crowded females. When a crowded female appeared grossly pregnant, it was removed and placed in a cage of its own. The others remained in the crowded group. All of the mice were sacrificed six weeks after the beginning of crowding. The reproductive tracts of the females were examined for embryos and implantation scars.

RESULTS

Infant weights at weaning, F_1 generation

The effects of crowding female mice on the mean weights of their progeny at birth and at weaning are summarized in Table 3. Mice nurtured by crowded females weighed 14.7% less at weaning than those nursed by females which had remained in segregated pairs ($P < 0.001$). Pups born to crowded mothers tended to weigh more at weaning than those born to isolated mothers, irrespective of which female nursed them, but the difference was not significant ($P < 0.10$). The mean weights of pups raised by their own mothers did not differ from those of pups raised by foster mothers ($P < 0.90$). There was no significant difference ($P < 0.40$) in mean weights of pups at birth between those born to crowded and those born to isolated females.

TABLE 3. COMPARISONS OF THE WEIGHTS OF YOUNG MICE IN THE F_1 GENERATION ACCORDING TO THEIR BIRTH OR NURTURING

Comparisons were made using the mean infant weight for each whole litter. See Figure 1 and text for the method of obtaining the whole litter values.

	No. of litters	Mean pup weight ± S.E. at weaning in grams	P diff.
Nursed by crowded female	56	7.84 ± 0.22	
Nursed by isolated female	56	9.09 ± 0.20	<0.001
Born to crowded female	56	8.80 ± 0.18	
Born to isolated female	56	8.31 ± 0.19	<0.10
Nursed by own mother	56	8.55 ± 0.19	
Nursed by foster mother	56	8.59 ± 0.09	<0.90
		at birth	
Born to crowded mothers	51	1.74 ± 0.049	
Born to isolated mothers	51	1.78 ± 0.055	<0.40

Weights at weaning vs. litter size, F_1 generation

Since the above comparisons disregarded litter size, it is worth examining the differences in weight at weaning with respect to litter size between the mean weights of pups raised by isolated and those raised by crowded females. These data are summarized in Table 4. The data for litters of 2, 3 and 4 young were combined because of the limited number of litters of these sizes. The number of litters of a given size raised by isolated females is not always equal to the number raised by crowded females, even though the litters were matched for size at birth (Table 4). These inequalities resulted from occasional differences in the survival of pups between a litter raised by isolated and one raised by the crowded female of a matched pair. The mean weights of the pups decreased with increasing litter size regardless of whether they were raised by crowded or by isolated females (Table 4, Fig. 2).

The mean weights of pups raised by crowded mothers were less than for their controls raised by isolated mothers for litter sizes of 5 or under, but the differences were small and not significant. However, in litters of 6 or more (except for litters of 9, which may be due to inadequate numbers for the degree of difference observed), the pups raised by crowded mice were significantly lighter at weaning than those raised by isolated mice. The mean weight of pups from litters of 6 or more raised by crowded females was 83% of the mean weight of pups from litters of the same sizes

TABLE 4. MEAN WEIGHTS OF MOUSE PUPS OF THE F_1 GENERATION RAISED BY ISOLATED OR BY CROWDED MOTHERS AND GROUPED ACCORDING TO LITTER SIZE

Each given mean value and its standard error were calculated from the mean pup weights for the individual litters. P values were calculated using Student's *t* test with the numbers of whole litters determining the number of degrees of freedom. Unequal numbers of litters for litter sizes of 5, 6, 7 and 8 were brought about by a differential mortality of the young between those raised by isolated or by crowded mothers, as all litters were paired for size and age at birth.

Litter size at weaning	Nursed by female	No. litters	Mean weight ±S.E. in grams	P diff.
2, 3, 4	crowded	6	9.89 ±0.74	N.S.
	isolated	6	10.73 ±0.30	
5	crowded	11	8.75 ±0.37	N.S.
	isolated	12	9.37 ±0.44	
6	crowded	19	7.73 ±0.30	<0.0001
	isolated	17	9.55 ±0.25	
7	crowded	5	7.12 ±0.39	<0.02
	isolated	4	8.77 ±0.33	
8	crowded	7	6.28 ±0.55	<0.02
	isolated	9	7.93 ±0.31	
9	crowded	5	7.90 ±0.46	N.S.
	isolated	5	8.73 ±0.80	
10	crowded	3	5.80 ±0.44	<0.06
	isolated	3	6.92 ±0.22	

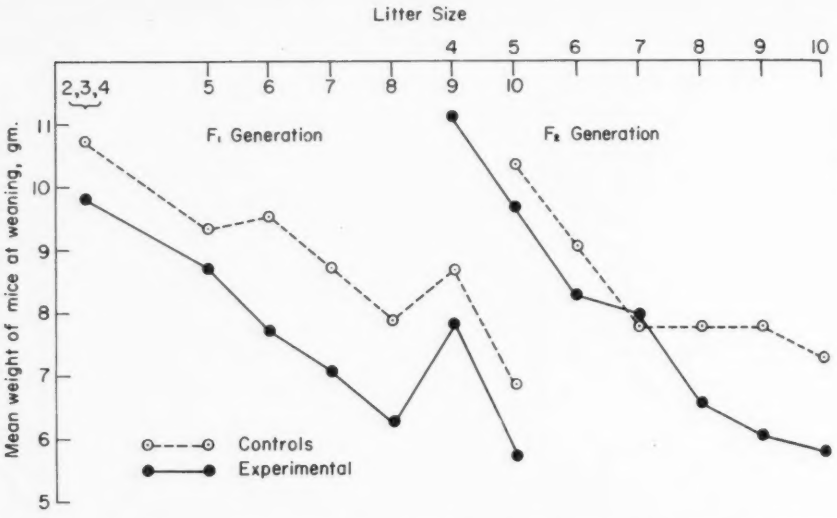

FIG. 2. Mean weights of progeny raised by crowded and by isolated mothers plotted against litter size (F_1 generation) and for progeny of mice raised by crowded mothers (F_2 generation). Mean values derived from the mean weights for each litter.

raised by isolated females, whereas in litters of 5 or less the mean weights of young raised by crowded females was 95% of that of young raised by isolated females. Regressions were fitted to the weights of infants on litter size. The slopes of regressions of the weight of the pups on litter size was −0.498 for those raised by crowded mice and −0.419 for those nursed by isolated mice, showing a more rapid decrease in weight with increasing litter size for pups nursed by crowded females than in their controls.

Weights at weaning, F_2 generation, and variations with litter size

Pups raised by females which in turn had been raised by crowded females weighed 8% less than their controls raised by isolated females ($P < 0.06$). The difference between the means was of questionable significance, but when the differences are examined with respect to litter size (Table 5, Fig. 2), it is clear that pups nursed by females which had been nursed by crowded mice weighed less than their controls for litters of 8 ($P < 0.05$), and 9 and 10 combined ($P < 0.05$). The weights of weaning mice from litters of 5, 6 and 7 did not differ significantly with respect to which mouse nurtured them, although the mean weights of progeny nursed by mice which were in turn nurtured by crowded females were less than those of their controls in every instance. If the data for litters of 8, 9 and 10 are combined, the mean weight of pups nursed by females which in turn were nursed by crowded females was 17.8% less than their controls ($P < 0.005$). There were no significant differences in the weights of progeny in the F_2

TABLE 5. MEAN WEIGHTS OF MOUSE PUPS OF THE F_2 GENERATION GROUPED ACCORDING TO LITTER SIZE

The females bearing and raising these progeny were raised either by crowded or isolated mothers. See text and legend for Table 3 for details.

Litter size at weaning	Nursed by mother which in turn had been nursed by females	No. of litters	Mean pup wt. ±S.E. in grams	P diff.
4	crowded	2	11.14 ±0.26	
	isolated			
5	crowded	2	9.67 ±0.57	<0.40
	isolated	2	10.36 ±0.14	
6	crowded	9	8.29 ±0.63	<0.30
	isolated	10	9.09 ±0.27	
7	crowded	2	8.00 ±1.70	<0.90
	isolated	2	7.80 ±0.10	
8	crowded	8	6.60 ±0.31	<0.05
	isolated	8	7.80 ±0.45	
9–10	crowded	7	5.86 ±0.31	<0.05
	isolated	8	7.44 ±0.66	
8, 9, 10	crowded	15	6.26 ±0.23	<0.005
	isolated	16	7.62 ±0.38	

generation with respect to the true parentage of mice in the F_1 generation. Therefore, the important factor in determining the differences in weights of progeny at weaning in the F_2 generation was whether or not the mouse nurturing them had been nursed by a crowded female.

The effects on the weights of the young are less marked in the F_2 than in the F_1 generation. The differences in mean weight between the experimental and control pups were significant only for litters of 8, 9 and 10 in the F_2 generation whereas litters of 6 or more weighed significantly less than their controls in the F_1 generation.

The litters measured may represent bias towards minimizing the effects of crowding as it is conceivable that these were produced by the females least affected by crowding.

Effects of crowding on reproduction

The results of crowding 10 male and 10 female mice in a single cage on reproductive performance, measured by live births, embryos, and uterine implantation scars, are summarized in Table 6. The presence of implantation scars showed that every female became pregnant, but that only 3 of the crowded females bore litters during the period of crowding or later. Therefore there was a marked intra-uterine mortality of implanted embryos. The fact that every female mouse became pregnant, even though she may have failed to carry the pregnancy to term, shows that the effect was not primarily on the fertility of the males. The intra-uterine loss

TABLE 6. SUMMARY OF DATA ON PRODUCTIVITY OF MICE CROWDED 10 PAIRS TO A CAGE COMPARED TO THEIR 10 CONTROL PAIRS

Note that all of the females became pregnant but that the crowded females exhibited a marked intra-uterine loss of young, reduction of implantation sites, reduction of litter size, and significant delay until the birth of first litters compared to the controls. Crowding produced a 75% loss in the number of young born.

	No. of pairs	No. litters born	Mean No. days to litter birth	Mean No. progeny per litter ± S.E.	No. females with placental scars	Mean No. scars per female
Crowded females	10	3	40 ± 1.0	7.67 ± 0.33	10	6.90 ± 1.37
Isolated females	10	10	26 ± 1.5	9.00 ± 0.75	10	11.00 ± 0.47

apparently occurred during the first half of gestation as none of the 7 without litters, but with implantation scars, ever appeared grossly pregnant. Apparently there also was some preimplantation loss in the crowded females as the mean number of implantation scars was significantly reduced from the control values obtained from the isolated littermate controls. It is not known whether the preimplantation loss was due to a failure of some of the fertilized ova to implant properly or to a reduced number of fertilized ova. A decrease in the fertility of the females is suggested by the 14 days greater mean time lapsing between exposure to males and the birth of the three litters of crowded mice compared to their isolated controls. These results suggest that crowding produced a serious loss of young due to intra-uterine mortality and probably to some decrease in fertility in female mice. These results probably explain the reason for the diminished productivity of crowded females in the preceding lactation experiments.

DISCUSSION

The above experiments demonstrate the adverse effect of crowding on lactation and reproduction in female albino mice, confirming and extending Chitty's observations on voles (18). Progeny raised by crowded females were adversely affected as were their progeny in turn.

It might be thought that crowding interfered with adequate nutrition of the females and that a resulting nutritional deficiency was responsible for the effects on the progeny. Several facts argue against such a suggestion. First, it has been shown that crowding has little if any effect on the body weight of adult albino mice (10, 11). Second, food in the form of pellets was generously spread all over the floor of the cage, so that any mouse was almost always in contact with food. There was therefore no competition for food or space at a feeding site. Daily observation further indicated that there was no interference with feeding or competition for food. Third, lactation began not less than three weeks after the mice were removed from the crowded cage (Table 2); so that there were three or more weeks of living in segregation with abundant food before lactation began. Finally,

the young of crowded mothers tended to weigh more at birth than those of isolated mothers and grew normally when nursed by isolated mothers. It therefore does not seem likely that the effects on the lactation of the crowded mothers, and hence on their offspring, can be attributed to inadequate nutrition.

Crowding apparently has no effect on the growth of young *in utero*, as there were no significant differences in weight at birth between the young of crowded and those of isolated mothers. Genetic effects are apparently ruled out by the fact that mice born to crowded and raised by isolated females actually tended to be heavier than those born to and raised by isolated mothers, and that mice born to isolated and raised by crowded females were smaller. Whether or not the young were raised by their own or a foster mother made no difference in their weights. Therefore the deleterious effects of crowding on the two succeeding generations of progeny must be attributed to inadequate lactation on the part of the originally crowded female and not to intra-uterine or genetic factors.

A difference in maternal behavior between previously crowded and isolated females was not detected in the course of daily observations. However, no detailed measurements were made to substantiate or disprove the impressions gained by the routine observations of maternal behavior. Brown (6) and Southwick (9) reported that interference with nursing and nest construction by other mice in the population was responsible for the deficient maternal behavior in their experiments. Interference by other mice was excluded from our experiments during the period of lactation by maintaining the mice as segregated pairs after crowding.

The only remaining alternatives to explain the decreased weight of young raised by crowded mice are quantitative and qualitative deficiences in the milk. The present experiments were not designed to distinguish between these two alternatives. One or both may have been operative, but a quantitative deficiency seems more likely, as in earlier experiments the stomachs of the young mice contained little or no milk when sacrificed (12).

Why females raised by previously crowded mice should in turn raise progeny smaller at weaning than their controls can only be conjectured. A specific factor in the milk is unlikely, and genetic factors have been ruled out. An explanation for the diminished size of the young mice in the F_2 generation could be that the effect of inanition resulting from deficient lactation affects the progeny of the first generation in such a manner that they, in turn, do not lactate adequately.

It has been noted that in every crowded population the number of mice at the end of six weeks of crowding was less than the original number, due mainly to the death of males, as fewer females than males died (Table 1). The number of male deaths varied from 3 to 14 per population, as shown by the number of pairs alive after crowding. The number of deaths is in a real sense a measure of the degree of social strife within a given

population, and the amount of strife is not necessarily a function of the absolute number of mice in a population. The aggressiveness of particular individuals as well as the degrees of difference in dominance and subordinance between the various members of the population are major factors in determining the amount of intraspecific strife in a given population. The bases for these statements will not be discussed here, as they have been covered in greater detail elsewhere (3, 8, 9, 12, 18, 20, 23, 25). It should be mentioned, however, that we have observed that the degree of stress engendered in a given population coincides with the amount of social strife better than with the actual number of animals (23). While no measurements of aggressive behavior or strife were made in the present experiments, it was noted that the amount of fighting observed corresponded to the degree of mortality, and these observations were consistent with those made on all of our experimental populations to date. It should be emphasized that fighting is rarely a direct cause of death (10, 11, 12, 23).

There was a direct correspondence between the number of pairs which survived crowding and the number of these which bore litters (Table 1). Evidently the amount of strife resulting in a given mortality rate affected reproduction in the surviving pairs to a comparable degree. The litters available for measurement were produced by less than half (68/140) of the crowded females, and not all of these litters survived until weaning. It is suggested that those females which produced young were physiologically less affected by crowding and therefore the least stressed. This suggestion is further supported by earlier experiments in which the dominant females in a group of mice have been reported to be better producers than their subordinate sisters (2, 3). Corresponding to these findings we have noted that adrenal weight, used as an index of stress, is least in dominant and greatest in subordinate mice (25). The present experiments offer some evidence that females having the largest litters were less affected by crowding than others. The differences between the mean weights of pups raised by crowded and those raised by isolated mothers were not proportionately as great for litters of 9 and 10 as they were for litters of 6, 7 and 8 in the F_1 generation (Table 4). These data suggest that females capable of producing large litters were also more capable of adequately nurturing their young than females bearing somewhat smaller litters. The results from the experiment with ten pairs of crowded mice show that reproductive failure in females surviving crowding is due primarily to intra-uterine mortality and secondarily to diminished fertility. All of the mice in this experiment became pregnant during the period of crowding, but only 3 gave birth to litters, and the mean size for these 3 litters was significantly less than for their controls. The onset of the first pregnancy also was considerably delayed compared to the control mice.

These experiments indicate that crowding can decrease productivity in female house mice by decreasing fertility, increasing intra-uterine mortal-

ity, and causing deficient lactation which results in diminished growth of the young. The young are themselves affected so that they in turn have smaller progeny, apparently as a result of deficient lactation. Data from populations of house mice in the laboratory (12) indicate that adversely affected progeny are less able to survive and reach maturity. Therefore crowding, or high population density, affects productivity immediately by decreasing fertility and increasing prenatal mortality, and at long range by depressing lactation with subsequent detrimental effects on the survival and productivity of progeny. It is likely that the severity of the effects is related to the degree of social strife and less closely to the actual number of animals interacting with one another (23). The experimental evidence indicates that recovery of reproductive competence from the effects of a period of high population density would necessarily be slow, possibly lasting several generations.

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ASSAY OF LACTOGENIC HORMONE[1]

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ABSTRACT

The micro or local method of lactogenic hormone assay has been examined using 15 common pigeons for each assay. The hormone was injected over the symmetrically opposite side of the crop-sac in some of the assays in order to compare responses resulting from two hormone concentrations. In 4 assays, only one side was injected.

The degree of proliferation of the crop-sac following injection of various levels of lactogenic hormone resulted in the establishment of a regression line, with a standard deviation of .0956, between log-dose per pigeon (X) and diameter (cm.) of proliferation obtained (Y) over a 31-fold dose range (.00072–.0224 mg. lactogenic hormone/bird). An index of precision (λ) of .11 was obtained. Responses resulting from injecting doses exceeding .0224 mg./bird or lower than .00072 mg./bird were difficult to measure.

THE discovery by Riddle and his co-workers (1–3) that the secretion of crop milk by pigeons and doves was under the control of a pituitary factor, later shown to be the lactogenic hormone, provided the basis for sensitive assay methods for the lactogenic hormone. One of the first was based upon crop weight (2, 4) where it was claimed a linear relationship existed between crop weight and log of dose. Later, methods were devised based upon the dose of lactogenic hormone injected subcutaneously or intramuscularly which would cause a minimal proliferative response in Silver King squabs (5) and in common pigeons (6). A local or "micro" method was introduced by Lyons and Page (7) and was improved upon by other investigators (8, 9). The "micro" method was found to be much more sensitive than other methods (10) and depended upon the degree of response obtained immediately beneath intradermally-placed injection sites. Reece and Turner (8) suggested that the response was directly related to dosage up to an arbitrary rating of 4, which was an area of proliferation 4 cm. in diameter. This latter method, as generally employed, is rather subjective since it depends upon individual interpretation of the degree of proliferation. Usually 2 or more individuals "rate" the responses in which case subjective error is somewhat diminished. Recently, a more

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objective criterion for estimation of the degree of stimulation in the local method of lactogenic hormone assay has been sought (11) by the comparison of the responses in the two crop-sacs of the same pigeon. It was concluded that potency of an unknown preparation could be estimated by injecting the unknown over one crop-sac and a standard over the other crop-sac in the same pigeon, resulting in an error of less than 30%.

In the present study we have re-examined the Reece-Turner micromethod of assay using a more objective method of determining proliferative responses and have established the usable range of the assay. Further, we have found the response related to the log-dose and have demonstrated that systemic carryover of hormone from one crop-sac to the other does not occur in this range. Thus, the estimation of the comparative potency of two unknown preparations in the same pigeon is possible.

MATERIALS AND METHODS

One hundred and fifty adult common pigeons of both sexes weighing 240–360 gm. were obtained and housed under uniform temperature conditions (78–80° F) in a room artificially illuminated during the normal daylight hours. They were fed Purina pigeon chow and water *ad libitum*.

Assay Procedure.—Fifteen pigeons were used for each assay. Lactogenic hormone[3] assaying 20 I.U./mg. was dissolved in pyrogen-free distilled water, then diluted to desired concentration. Fresh solutions were prepared for each assay. Two solutions to be tested were injected intradermally in 0.1 ml. volume daily for 4 days over the symmetrically opposite sides of the crop-sac of each of 15 pigeons in all but 4 assays. In these, only one side was injected. The injection areas, which were located over the geometrical center of the crop-sacs, were previously marked with a non-toxic dye to insure injecting in the same area each day. Twenty-four hours after the last injection each pigeon was killed, the entire crop removed and cut in halves. Each half then was stretched fully by two persons over a light source while a third person matched the area of response as closely as possible with one of 8 plastic gauges which ranged in diameter from 0.50 to 4.00 cm. Each gauge consisted of a thin circle of clear plastic connected to a handle. By this method the proliferative response could easily be seen and accurately measured, for in the vast majority of crop-sacs, circular proliferative responses were obtained.

Standard deviation of Y (mean diametric response at dose X) was determined by the formula

$$SY = \sqrt{\frac{\Sigma Y^2 - a\Sigma Y - b\Sigma XY}{n - 2}} \quad (12).$$

Results.—The degree of proliferation of the pigeon crop-sac following the injection of various levels of lactogenic hormone results in the establishment of a regression line with a standard deviation of .0956 between log dose used per bird (X) and diameter of proliferation obtained (Y) over a 31-fold dose range of lactogen (.00072–.02240 mg./bird) (Figure 1). An index of precision (λ) of .11 was obtained. From the data in Table 1 it can be seen that the average diametric response resulting from any dose

[3] We are indebted to the Endocrinology Study Section of the National Institutes of Health for the gift of ovine Lactogenic Hormone.

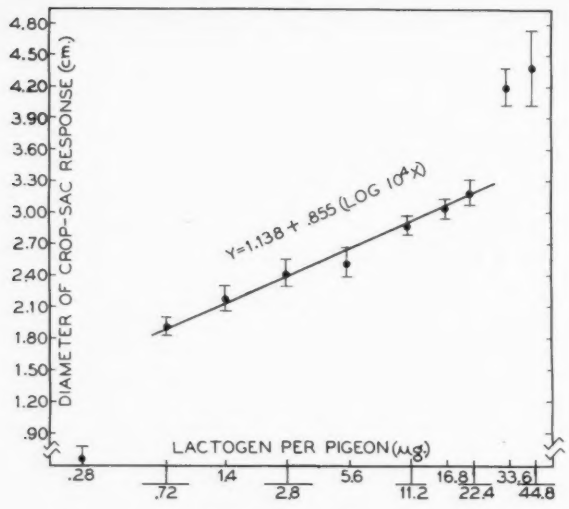

FIG. 1. Pigeon crop-sac response to lactogenic hormone (1 mg. = 20 I.U.) by local or "micro" method of assay using the common pigeon as the assay animal. Standard deviation of Y = .0956 cm.

level corresponds very well with the median response. Further, a normal frequency distribution of values, accompanied by a rather uniform standard error, is obtained for each assay within the regression line. Responses obtained with doses exceeding .0224 mg./bird were difficult to measure accurately since in most instances the entire crop-sac had undergone proliferation and, with the greater dose used (.0448 mg./bird), a general thick-

TABLE 1. DATA COMPARING RESPONSES IN THE TWO CROP-SACS OF THE SAME PIGEON USING VARIOUS DOSES OF LACTOGENIC HORMONE (1 MG. = 20 I.U.)
Fifteen pigeons used per assay

Lactogen assay	Total lactogenic hormone injected per bird over one crop-sac (mg.)	Average diametric response (cm.)	Median diametric response (cm.)	Average pigeon weight (g)	Total lactogenic hormone injected per bird over opposite crop-sac (mg.)	Ratio of dosage in same bird
1	.00028	.66 ± .12	.50	320.2	—	—
2	.00072	1.92 ± .08	1.96	316.5	.03360	47x
3	.00140	2.18 ± .15	2.20	306.7	—	—
4	.00280	2.44 ± .12	2.60	320.2	.03360	12x
5	.00560	2.42 ± .12	2.40	311.6	.01120	2x
6	.00560	2.62 ± .18	2.80	314.2	.01680	3x
7	.00560	2.54 ± .15	2.56	304.1	.02240	4x
8	.01120	2.88 ± .09	2.76	311.6	.00560	2x
9	.01680	3.00 ± .10	3.00	314.2	.00560	3x
10	.01680	3.10 ± .06	3.06	325.2	.02240	1½x
11	.0240	3.18 ± .14	3.30	304.1	.00560	4x
12	.0240	3.26 ± .10	3.40	325.2	.01680	1½x
13	.03360	4.12 ± .09	4.16	316.5	.00072	47x
14	.03360	4.06 ± .20	4.00	313.2	—	—
15	.03360	4.32 ± .16	4.20	323.1	.00280	12x
16	.04480	4.40 ± .36	4.00	296.7	—	—

ening of the crop-sac had commenced. Response to the lowest dose tested (.00028 mg./bird) resulted in minimal proliferation. Two of 15 pigeons receiving this dose failed to respond at all, while the remainder responded with a small, but measurable proliferation. There was no apparent effect of a high dose injected over one crop-sac upon the response obtained with a lower dose injected over the opposite crop-sac since, in the cases tested, the responses fell well within the regression line. Further, in 4 assays in which only one crop-sac was injected, no response was obtained in the uninjected side.

Discussion.—There are several variants which affect the response of the pigeon to lactogenic hormone (13). The important ones, as long as injection route and volume are standard, are weight, age, race, season and temperature. We have, in the present study, attempted to reduce these variants by employing common pigeons of a particular weight range (and, therefore, presumably age) and by housing them under uniform conditions of temperature and lighting. The common pigeon, though not as desirable as a pure strain (4, 14), lends itself very well to lactogenic hormone assays for, in addition to being available in great quantities in most areas, is less costly and adapts itself well to confinement.

One of the principal criticisms of the "micro" method of assay, particularly where responses other than minimal were obtained, e.g., Reece-Turner method (8), has been the subjectivity and subsequent lack of quantitativeness. In the present investigation we have attempted to increase the uniformity and objectivity of the method. A good correspondence of mean and median values resulted along with a rather uniform standard error for each assay within the regression line.

Reece and Turner (8), using the "micro" method, defined one unit of lactogenic hormone as the amount which caused a proliferation of 2 cm. in diameter in the common pigeon and suggested the area of response was directly related to dosage up to a rating of 4, an area 4 cm. in diameter. Meites and Turner (15) have shown that the most quantitative results could be obtained by injecting enough hormone to induce a rating of 2 (an area of response 2.8 cm. in diameter), or less. The results of the present study suggest repeatable quantitative results are obtained over a 31-fold dose range of lactogenic hormone which induced responses of 1.92–3.26 cm. in diameter. However, doubling the dose did not result in a doubling of response area but rather a log-dose response situation existed. A high index of precision ($\lambda = .11$) was obtained with our method of assay. We feel, therefore, our local or "micro" method of lactogenic hormone assay, employing the common pigeon as the assay animal, to be a convenient and an accurate method for expression of lactogenic hormone potency. Results obtained from assay of preparations of unknown potency then may be expressed in terms of mg. standard, or, as I.U. simply by multiplying the value of X (mg.), obtained from the regression equation, by 20 (20 I.U./mg.)

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PITUITARY LACTOGENIC HORMONE CONCENTRATION AND MILK SECRETION IN LACTATING RATS[1]

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ABSTRACT

The role of the lactogenic hormone in maintaining lactation in the rat has been studied by comparing milk secretion with the change in concentration of pituitary lactogenic hormone resulting from nursing on each of days 2, 6, 10, 14 and 21 postpartum.

Milk yield per timed nursing, litter body weight and intensity of milk secretion were found to increase throughout lactation, though a slight lag in all occurred on day 14.

Maternal body weights were rather uniform but maternal pituitary weight per 100 g. decreased steadily from beginning to end of lactation.

Pituitary lactogenic hormone levels of lactating rats, which were isolated from their young for 10 hours to standardize the influence of nursing stimuli (prenursing level), and of rats allowed to nurse 30 minutes after isolation (postnursing level) was determined by the common pigeon micro-method of assay. The prenursing level of lactogenic hormone increased from the 2nd to the 6th day postpartum, then declined toward the end of lactation. The fall in hormone concentration as a result of 30 minutes nursing stimuli was the greatest on days 6 and 14 (95% and 90%, respectively). The smallest decline occurred on day 21 (4%), the time of maximum milk secretion. The reason for this is unknown.

IT IS well known that lactogenic hormone secreted by certain acidophil cells of the anterior lobe of the pituitary gland plays a vital role in the initiation and maintenance of milk secretion (1). The number and secretory activity of acidophilic cells normally increases in late pregnancy and following parturition in the rat (2), then gradually declines as lactation advances. A parallel increase and decline in the lactogenic hormone content of the rat pituitary gland has been observed (3). It is believed the initial increase in pituitary lactogenic hormone is due to estrogen (4) which reaches a peak in the blood and urine near parturition but, as the estrogen level subsides after parturition, maintenance of lactogenic hormone secretion becomes dependent upon the regular application of milking or nursing stimuli. Since the investigations of Reece and Turner (5), evidence has accu-

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mulated proposing that nervous stimuli resulting from milking or nursing are transmitted to the anterior lobe, causing a release of lactogenic hormone into the blood-stream. Recently it has been shown that such discharge occurs very rapidly in the lactating rat once the stimulus of nursing has been applied, but subsequent restoration of the prenursing level is a slower process (6). In spite of regular application of such stimuli, the pituitary level of lactogenic hormone in animals tested declines with the advance of lactation (3, 7–9) though in species such as the rat, the lactation curve is still rising (10, 11). Since the absolute pituitary level of a hormone perhaps is not the best indication of secretory activity, it seemed of interest to further evaluate the role of the lactogenic hormone during the lactation cycle by the comparison of milk secretion with the fall in pituitary lactogenic hormone concentration which occurs during timed nursings. In the present investigation we have shown 30 minutes nursing elicits a fall of up to 95% in the pituitary lactogenic hormone level during most of the lactation period, but toward normal weaning time only small quantities apparently are released due to the nursing stimulus, though milk secretion is still very intense.

MATERIALS AND METHODS

Adult primiparous lactating rats weighing 275–325 g were housed in individual cages under conditions of uniform temperature (78–80° F) in a room artificially illuminated during the normal daylight hours. They were fed Purina lab chow and water *ad libitum*. Shortly after birth each litter was adjusted to 6 young and, whenever possible, were equally distributed as to sex. The following procedure was used on each of days 2, 6, 10, 14 and 21 postpartum: Each of 30 lactating rats was isolated from her litter for 10 hours; at the end of this time mother weights were recorded. Fifteen then were killed. The remainder were united with their young, allowed to nurse exactly 30 minutes, then killed immediately. After death of the mothers, their pituitary glands were removed, weighed individually and frozen until assayed for lactogenic hormone. Five pituitary glands were pooled for each assay. These were crushed in an agate mortar, suspended in a measured amount of distilled water and injected intradermally over one of the crop sacs of 15 mature adult common pigeons of both sexes weighing 260–340 gm. Distilled water solutions of standard lactogenic hormone[3] were injected similarly over the opposite crop sacs in some of the assays. Resulting crop gland responses were measured and converted to mg. lactogenic hormone by the recent micromethod of assay of Grosvenor and Turner (12). The regression equation from the assay procedure (12) was employed in the conversion of crop gland responses obtained in the present study since in every instance the average crop gland response to the standard was ± 5–10% of that produced with the corresponding dose of the standard in the original assay.

After each litter had been nursed 30 minutes, they were weighed, killed by decapitation and their stomach contents removed and weighed.

RESULTS

It was observed most litters ceased active sucking with approach of the end of the 30 minutes nursing period. They either would fall asleep while

[3] The lactogenic hormone (NIH-SP-1) is a highly purified sheep preparation estimated to contain 20 I.U./mg., being distributed by the National Institutes of Health.

TABLE 1. MILK REMOVAL BY NURSING RATS AT INTERVALS DURING LACTATION

Day of lactation		No. of rats	Average Litter weight (gm.)	Milk weight (gm.)	Milk weight / Litter weight (%)	Mother weight (gm.)	Pituitary weight (mg.)	Pituitary weight 100 gm.
2		15	40.5	—	—	282.6	12.1	4.30
		15	39.9	1.44	3.6 ± .29[1]	294.1	12.5	4.25
	Mean		40.2			288.4	12.3	4.28[5]
6		15	72.0	—	—	295.7	11.9	4.06
		15	77.5	3.10	4.0 ± .36	293.1	11.6	3.96
	Mean		74.8			294.4	11.8	4.01
10		15	126.7	—	—	298.7	11.6	3.91
		15	127.6	6.40	5.0 ± .33[2]	308.7	11.6	3.76
	Mean		127.2			302.7	11.6	3.84[6]
14		15	148.4	—	—	289.9	10.5	3.62
		15	158.4	6.80	4.3 ± .45[3]	292.5	9.3	3.18
	Mean		153.4			291.2	8.9	3.40
21		15	257.5	—	—	309.5	10.3	3.33
		15	262.4	15.50	5.9 ± .38[4]	314.8	10.0	3.18
	Mean		260.0			312.2	10.2	3.26[7]

	Student's "t"	Probability
1–2		.005
1–3		.2
1–4		.005
2–3		.2
2–4		.2
3–4		.005
5–6		.005
6–7		.005

attached to the mother or the mother would disengage herself from her young. The amount of milk thus obtained by litters of 6 young and the weight of the young increased steadily throughout lactation, though a slight lag occurred in both between days 10 and 14 (Table 1). The weight of milk obtained, expressed as per cent litter body weight, similarly increased during lactation with the exception of an insignificant drop which occurred on day 14 (Table 1). The per cent yield did not vary significantly between days 2, 6 and 14, but a significantly greater yield over that of days 2 and 6 occurred on day 10. The maximum per cent yield, obtained on day 21, was significantly increased over that of days 2, 6 and 14, but not that obtained on day 10. It is of interest to note maternal body weights were rather uniform while milk yields were increasing. However, maternal pituitary weight per 100 g. body weight decreased steadily from the beginning to the end of lactation (Table 1). This decrease was highly significant by the 10th day postpartum.

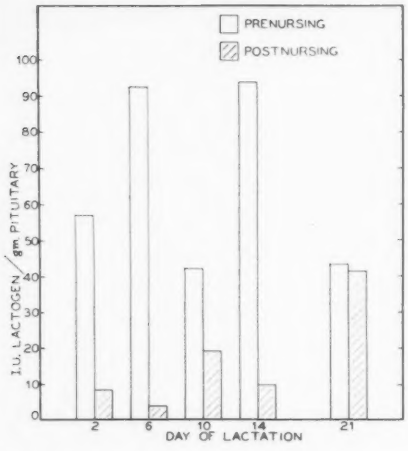

FIG. 1. Fall in pituitary lactogenic hormone concentration as a result of 30 minutes nursing stimuli (postnursing level) following 10 hours isolation of mother and young (prenursing level). Each point represents mean of 15 pituitary glands.

The pituitary lactogenic hormone level of lactating rats which were separated from their young for 10 hours to standardize the influence of nursing stimuli (prenursing level) was highest on days 6 and 14 (Fig. 1). Also, the fall in pituitary lactogenic hormone level as a result of nursing stimuli was the greatest on these days (95 and 90%, respectively). The smallest decline due to nursing was 4% and occurred on day 21.

DISCUSSION

It is known (10, 11) the amount of milk produced by lactating rats increases steadily throughout lactation, though the incidence of nursing decreases as lactation advances (13). It would be expected, therefore, that older, heavier litters would obtain more milk per timed nursing. This has been confirmed in the present study. Litter weight also increased with increased milk yield indicating greater milk production as lactation advanced since the heavier the litter, the more milk must be secured to satisfy increasing energy requirements for growth and maintenance. Other conditions being the same, the amount of milk obtained per timed nursing, expressed as per cent litter body weight, then becomes a rough measure of milk secretion. On this basis, results of the present investigation show milk secretion increased during lactation, though an unexplained decline occurred on the 14th day postpartum. A similar depression has been observed in lactating mice (14). The increase in the prenursing pituitary lactogenic hormone level from the 2nd to the 6th day postpartum and subsequent decline toward the end of lactation observed in the present study, is in accord with results obtained with rabbits (7, 9), mice (8) and rats (3). Estrogen has been demonstrated to increase pituitary weight in rats and to evoke elevation in pituitary lactogenic hormone levels (15), so the possibility arises, decline in pituitary weight and reduction in prenursing lactogenic hormone levels may be due to the reduced estrogen secretion which occurs during

actation in the rat. As maintenance of milk secretion is undoubtedly a response to the coordinated actions of several pituitary hormones, a suboptimal secretion of any one of them, therefore, could result in diminished milk secretion. Though the prenursing pituitary lactogenic hormone concentration declined in late lactation in the rat, such decline should not be interpreted as suboptimal hormone output. If this were so, milk secretion would have been expected to diminish. Instead, heightened milk secretion was associated, in the present study, with lowered pituitary lactogenic hormone levels. Similarly, no coinciding peak between the magnitude of fall of the pituitary lactogenic hormone level due to nursing and lactation was apparent. Ironically, maximal lactation was associated with a minimal drop in hormone concentration on the 21st day of lactation. The reason for this is not clear, though there are several possibilities: The slight reduction from the prenursing level on day 21 may indicate that a conditioning of reflex mechanism for lactogenic hormone release has occurred. This could result in periodic discharge of hormone perhaps due to external stimuli such as sight and smell of the young, or to stimuli originating from within the animal such as milk pressure. Another possibility is that continuous secretion and discharge of lactogenic hormone may occur at this stage of lactation in the rat which is independent of application of nursing stimuli. If either of these possibilities are true, the total amount of lactogenic hormone discharged, e.g., per day, therefore, might be equivalent to, or in excess of, that released within the same interval during earlier stages of lactation. A somewhat remote explanation may be that a gradual reduction in efficiency of the mammary-pituitary reflex for lactogen release occurs as lactation advances. If this is so, it must be assumed the small amount of hormone released as a result of nursing stimuli is sufficient to allow for secretion of the large quantity of milk produced at this time.

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HORMONALLY-INDUCED LACTATION IN HYPOPHYSECTOMIZED RATS[1]

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ABSTRACT

Long-Evans rats hypophysectomized on the 12th day of pregnancy showed a small amount of milk in their mammary glands on the day of parturition. Untreated, such rats resorbed this milk and were unable to supply enough to their own or foster young for even one day's maintenance. Mammotropin (MH) and cortisol proved to be adequate substitution therapy in raising the milk production in such animals to approximately 50% of normal, and in maintaining it at that level. During these tests the mothers lost about 1 gm. of body weight daily while each of six pups gained that amount. It was unnecessary to inject oxytocin for milk conduction in these rats, although the degree to which the endogenous secretion of this hormone had returned was unknown. No good evidence was obtained to prove that either somatotropin or thyroxin, when added to an MH and cortisol regimen, improved the lactational response. No one of the mentioned hormones supported lactation nor did any combination that excluded both MH and cortisol. These two hormones are not only important in initiating milk secretion, but also in maintaining lactation in the rat.

IN 1938 (1) it was reported that lactation could be induced in rats hypophysectomized one day ante-partum by injections of a lactogenic hormone preparation containing high unitages of mammotropin (MH, prolactin) and adrenocorticotropin (ACTH). The young did not obtain enough milk to be maintained although there was ample in the glands of most of the mothers. This failure has been partially attributed to an inadequate supply of oxytocin from the hypothalamic source interrupted at hypophysectomy and from the extracts used. Also, in retrospect the doses of MH and ACTH may be said to have been excessive.

In 1939 and 1940, Gomez (2, 3) reported in two abstracts that he was able to induce lactation in hypophysectomized rats injected with pituitary and adrenocortical extracts only when pituitrin was added to the regimen; and even then lactation was subnormal. Since then, there have

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been few attempts to quantitate lactational responses to hormones in hypophysectomized animals. Recently Cowie (4, 5) has developed a test using rats hypophysectomized on the 4th day post-partum; and Binarningsih *et al.* (6) have reported their findings in rats hypophysectomized in mid-pregnancy and studied after parturition. In the meantime, many experiments have been performed in which milk secretion has been induced in hypophysectomized animals without attempts being made to study the full lactational response which also involves milk removal (see reviews , 4, 8). Lactogenesis in response to hormones has been studied in the simplest form (mammary cultures) by Elias (9), and in what must be considered the most complex forms, namely ruminants in declining lactation under the influence of all of their own and fetal hormones including those of the placenta (7, 10, 11).

EXPERIMENTAL PROCEDURE

In these experiments information was sought concerning the whole nursing performance of hypophysectomized Long-Evans rats under different hormonal regimens during the 10-day period following a first pregnancy. All animals delivered living or dead young at the expected time or one to three days later. The mammary glands continue developing in such rats hypophysectomized on the 12th day of gestation (12, 13), and a small amount of milk may be found in these glands for a short time after parturition. There is never enough to sustain even one young; and if no treatment is instituted, the milk resorbs in one to two weeks. It should be emphasized, therefore, that in this study hormones were given to enhance or maintain milk secretion already initiated.

Of any 12 rats prepared for the lactation test, it is rare to have even one succumb to hypophysectomy. Usually not more than one resorbs its conceptus; and one may become "toxic" and die after having been unable to deliver its young. And lastly, it is not unusual for one of the original dozen to neglect its own or foster young, and thus disqualify itself as a suitable test animal. It was learned, as previously shown (14, 15), that a hypophysectomized rat with no hormonal substitution may act as solicitously toward its own or foster young as do normal mothers; and that neglect may be exhibited in spite of the pituitary's presence or hormonal induction of an abundant milk secretion. It seemed best to exclude the few totally neglectful mothers regardless of whether they belonged to the hormone-injected or control groups, and regardless of whether their glands were empty or full of milk. They were treated and studied as other rats in their group for the usual 10-day period, but they could not be used in quantitative tests for milk formation and delivery.

These poor nurses could usually be recognized as such immediately after or even before parturition. They did not retrieve well or at all; they were slovenly about nest building or neglected it; they seemed irritable with their own or foster pups, and left them to die within a day or two, cold and unfed. In contrast to these, the great majority of the hypophysectomized rats whether treated or not exhibited good maternal behavior, permitting their nipples to be drawn out to great lengths when there was no milk, and often carrying the pups to the food cups hoping that they might serve themselves.

The advantage of rats hypophysectomized in mid-pregnancy over those operated upon during lactation is largely in the avoidance during the test of such indisposition as may follow a traumatic procedure. Furthermore, the oxytocin-secreting mechanism of the hypothalamus becomes restored in the 10–14 day interval to such an extent that no oxytocin need be injected to ensure milk movement along ducts. Hypophysectomy in

mid-gestation also made it possible to deplete the test animal of the pituitary factors and the target organ hormones. The placental factor that imitates MH in its luteotropic and mammary stimulating activities remains active; and accounts for the maintenance of pregnancy and the mammary apparatus. However, the corpora lutea degenerate by the end of pregnancy; and no new ones form as is the case when hypophysectomy is delayed until a day or two after parturition.

On the day of parturition each mother with her own or a foster litter was caged separately; and nesting, retrieving and nursing behaviorisms were noted. A check was made each day for the presence of milk in the maternal glands and the pups' stomachs, and, daily, individual weights were recorded. The first 5 days were used as an adjustment period, whereas the second (and in a few cases the third) 5-day periods were used for quantitating the lactational response. The pups were used as a means of withdrawing the milk and quantitating its effect on the basis of body weight increment; and this was best accomplished by keeping 6 active foster pups (initially 6–7 gm. in weight) with the mother constantly throughout the 6–11 day period. These pups were weighed at the beginning of the experiment with their alimentary tracts full of milk from their own mothers—as may be observed through the skin; and therefore the daily weight increment represented gain in tissue weight for the most part, but not entirely. In a positive response there was noted a steady climb in the body weight curve although the mean daily increments remained fairly constant. During the course of this experiment it was noted that 14 normal rats supplied their first litter of 6 with enough milk for them to show mean daily body weight increments of 1.4 gm. for the first 5-day period and 2.2 gm. for both the second and third 5-day periods. There, as in the experimental animals, one can only estimate the amount of milk ingested on the basis of body weight increment. Brody and Nisbet (16), and Cowie and Folley (17) among others have done this; and the latter have estimated that the mean daily gain in weight per litter over the 6–11 day period ("the litter growth index") is roughly 50–60% of the true milk yield. One does not assume from this that the amount of milk required by a 6-day-old pup to increase in weight from 13 to 15 gm. would be as much as that needed by an 11-day-old one to increase from 23 to 25 gm. The latter must maintain a much larger tissue mass, and add to it an equal daily increment—a feat that might possibly be accomplished by the same amount of milk of better constitution and more efficiently utilized. This point is important in judging the results of experiments in which two 5-day periods [6–11, 11–16] were used in comparing different hormonal regimens in the same mother-litter combinations.

The MH was prepared from ovine pituitaries, and contained approximately 30 I.U. per mg. There was less than an estimated 1% contamination of the other anterior and posterior lobe hormones; and positive lactogenic responses to these MH preparations were duplicated with solutions boiled to destroy trace contamination with all of the other hormones but ACTH. The somatotropin (STH) was prepared from bovine anterior lobes. It contained approximately 2% of MH and less than an estimated 1% of other hormonal contaminants. The thyroids and adrenals of the test rats in these experiments showed no histologic evidence of stimulation after 10 daily doses of 5 mg. of MH, but slight repair after 10 daily doses of 2 mg. of STH. Different levels of MH, STH and either cortisol acetate or prednisolone acetate were injected daily subcutaneously alone and in various combinations. The injections were started on the day of parturition and continued for 10 days or more. Dosage was based upon figures and results published previously (18, 19) on the initiation of milk secretion in hypophysectomized male and female rats of this strain.

RESULTS

In Table 1 are listed some of the hormonal regimens that have been

TABLE 1. NEGATIVE LACTATIONAL RESPONSE IN HYPOPHYSECTOMIZED RATS INJECTED WITH MAMMOTROPIN, SOMATOTROPIN OR CORTISOL ALONE AND IN CERTAIN COMBINATIONS

Group	No. of mothers	Hormones (mg./day)			Lactation	Milk in Glands*
		MH	STH	Cort.		
1	14	–	–	–	–	–
2	5	–	–	1.0	–	–
3	2	–	–	0.4†	–	– to +
4	3	–	2.0	–	–	– to +
5	2	2.5	–	–	–	+
6	3	5.0	–	–	–	+
7	3	10.0	–	–	–	+
8	4	–	2.0	2.0	–	– to ++
9	2	–	1.0	0.4†	–	– to +
10	3	5.0	0.5	–	–	+ to ++
11	8	5.0	1.0	–	–	+
12	2	10.0	2.0	–	–	– to +

* As seen microscopically after necropsy on the day after 10 daily injections.
† Prednisolone acetate instead of cortisol acetate.

found inadequate in lactational tests. MH, STH, cortisol, prednisolone, MH plus STH, STH plus cortisol or prednisolone were ineffective in a limited range of doses in supporting even a single pup. A trace of milk present in these animals and uninjected controls after parturition and before injections were started, sometimes persisted for the 10-day observation period. It could only be demonstrated by careful manipulation of the glands through the skin and by microscopic study of the tissue taken at necropsy. Some of the glands from these hormone-treated rats showed better parenchymal maintenance than the uninjected controls. This might indicate a residual or slightly stimulated adrenocortical function in cases receiving MH or STH without cortisol and slight activity of the trace contamination. Hormonal doses that maintain mammary alveolar cells or stimulate them minimally to secrete milk must be considerably lower than those required for a litter-sustaining lactation.

In Tables 2 and 3 may be seen the results obtained with a small range of dosages of MH plus cortisol and MH plus STH and cortisol respectively. For comparative purposes the prednisolone acetate (delta-1 cortisol acetate) may be considered approximately three times as potent as the cortisol acetate in glucocorticoid activity. The doses of both cortisols used antagonize the body weight increasing activities of STH; and so the doses of the latter were kept fairly high to ensure a slight maternal body weight gain during the 10-day period. Any beneficial effect on maternal weight that might have been expected from the higher doses of MH would have to be considered neutralized by the cortisol. The mothers injected with MH plus cortisol usually lost weight. In the day-to-day weighings, wide fluctuations were noted in maternal body weights due, mainly, to the degree of fullness of the mammary glands. However, the practice of weighing the mothers in the late afternoon was also followed in the case of 13 normal rats during the same period of lactation; and attempts were made to

TABLE 2. LACTATIONAL RESPONSES IN HYPOPHYSECTOMIZED RATS INJECTED WITH MAMMOTROPIN AND CORTISOL

Group	No. of mothers	Pups per litter	Hormones (mg./day)		Average body weight change (gm./day)	
			MH	Cort.	Mother	Pup
13	3	6	10.0	0.4†	−1.3	+1.1 (0.6–1.6)*
14	7	6	5.0	0.4†	+0.3	+0.8 (0.4–1.2)
15	7	6	5.0	1.0	−1.8	+0.9 (0.5–1.4)
16	4	4	5.0	0.2†	−0.6	+1.2 (0.8–1.2)
17	4‡	4	5.0	0.2†	−0.4	+0.9 (0.3–1.4)
18	3	6	5.0	0.5	−0.4	+0.9 (0.5–1.2)
19	3	6	3.0	1.0	−1.0	+0.5 (0.3–0.8)
20	3	6	2.5	0.4†	−1.1	+0.8 (0.4–1.0)

* Range of mean pup body weight increments in different litters.
† Prednisolone acetate used instead of cortisol acetate.
‡ Also received 5 μg. l-thyroxin daily which raised O_2 consumption to normal range.

imitate as closely as possible the 1–2 gm. daily gains in the normal mothers by giving adequate STH to some experimental groups.

Most of the lactational responses in rats that received MH plus one of the cortisols fall close to 50% of normal. The wide range of mean weight responses in the different litters, as shown in the tables and the paucity of litters make it difficult to detect differences in lactation that could be attributed to dosage of MH or cortisol. From these results it may only be said that a daily dose of 5 mg. of MH and 1.0 mg. of cortisol (0.4 of prednisolone) is adequate to establish a level of lactation at approximately 50% of normal.

Of the 11 groups of mothers that received STH in addition to MH plus cortisol, three (Groups 22–24) seemed to show slightly better responses.

TABLE 3. LACTATIONAL RESPONSES IN HYPOPHYSECTOMIZED RATS INJECTED WITH MAMMOTROPIN, SOMATOTROPIN AND CORTISOL

Group	No. of mothers	Pups per litter	Hormones (mg./day)			Average body weight change (gm./day)	
			MH	STH	Cort.	Mother	Pup
21	5	6	10.0	2.0	1.0	+3.8	+1.0 (0.3–1.9)*
22	5	6	10.0	2.0	2.0	+0.9	+1.8 (0.9–2.7)
23	5	6	10.0	1.0	1.0	+0.9	+1.5 (1.1–2.0)
24	5	6	5.0	1.0	1.0	+1.0	+1.6 (0.9–1.7)
25	3	6	5.0	1.0	0.4†	+1.6	+0.8 (0.1–1.4)
26	4‡	4	5.0	1.0	0.2†	+2.2	+0.9 (0.4–1.5)
27	4	6	5.0	4.0	0.4†	+5.2	+0.6 (0.3–0.8)
28	6	6	5.0	0.5	0.5	+2.2	+0.6 (0.2–0.9)
29	3	6	5.0	0.2	1.0	−0.8	+0.5 (0.3–0.9)
30	4	6	2.5	1.0	1.0	+1.0	+1.0 (0.8–1.3)
31	3	6	2.5	1.0	0.4†	+2.2	+0.9 (0.8–1.3)

* Range of mean pup body weight increments in different litters.
† Prednisolone acetate used instead of cortisol acetate.
‡ Also received 5 μg. *l*-thyroxin daily which raised O_2 consumption to normal range.
13 normal mothers showed a mean weight increment of 1 gm./day from day 5 to 10. Their litters of 6 showed a mean weight increment of 2 gm./day/pup.

Two of these groups of 5 rats were on the high dose of 10 mg. of MH and 1 or 2 mg. of STH. On the whole, the addition of STH improved the condition of the mothers, but this was not uniformly reflected in the better growth of the pups. To feel secure in attributing lactational improvement in the range of 20% to added STH as has been done in cows (20, 11, 7, 21), groups of 20 mothers may have to be used in the rat test.

In comparing the responses to lactogenic regimens with and without STH, the wide range of mother-litter variations may be controlled as shown in the data obtained from Groups 13, 14, 16 and 20 and presented in Table 4. Having obtained figures on maternal and pup body weight change in response to MH plus prednisolone during the usual 6–11 day

TABLE 4. LACTATION TESTS PERFORMED IN SEQUENCE ON THE SAME HYPOPHYSECTOMIZED RATS INJECTED WITH THE SAME OR DIFFERENT HORMONAL REGIMENS

Group	No. of mothers	Pups per litter	5-day period	Hormones (mg./day)			Av. body wt. chg. (gm./day)		Daily increment/pup			
									Litter No.			
				MH	STH	Pred.*	Mother	Pup	1	2	3	4
14	3	6	1st	5.0	—	0.4	−1.1	+1.0	0.7	1.2	1.2	—
			2nd	5.0	1.0	0.4	+2.2	+1.1	0.8	1.4	1.2	—
20	3	6	1st	2.5	—	0.4	−1.1	+0.8	0.9	0.4	1.0	—
			2nd	2.5	1.0	0.4	+2.1	+0.9	1.2	0.5	1.2	—
16	4	4	1st	5.0	—	0.2	−0.6	+1.2	0.8	0.8	1.9	1.2
			2nd	5.0	1.0	0.2	+2.5	+1.2	0.4	1.0	2.0	1.4
13	3	6	1st	10.0	—	0.4	−1.3	+1.1	0.6	1.2	1.6	—
			2nd	10.0	—	0.4	+1.5	+1.4	0.9	1.6	1.7	—

* Prednisolone (delta-1 cortisol) acetate; about 3× as potent as cortisol acetate.

period, 1 mg. of STH was then added to the regimens and the daily weight changes were recorded for the 11–16 day period. In Group 13 the response to the same MH plus prednisolone dosage was followed through the two 5-day periods. All of the 26 litters studied except one (No. 1 of Group 16) show a similar or slightly better daily weight increment; and as in the earlier experiments this averaged about 1 gm. per day. Whether the mothers lost 1 gm. per day or gained 2 gm. due to added STH, the litters gained at approximately the same rate or at best improved to the extent of 10–20% in the second period. As suggested above, about double the body mass was being maintained during the second period as compared with the first, and a correction for this factor would favor the second tests without necessarily crediting STH with the enhancement of milk production. The order of regimens would have to be reversed in order to control improved mother-litter relationship, differences in oxytocin secretion, etc.

Of special interest were two groups of mothers that received 5 μg. of thyroxin daily during the test in order to restore their metabolic rates to the normal range. These were the only experiments in which 4 instead of 6 pups per litter were used. The young of 4 litters in Group 16 (Table 2) with mothers on 5 mg. of MH and 0.2 mg. of prednisolone gained 1.2 gm.

per day, whereas those of Group 17 with mothers on the same hormones plus 5 μg. of thyroxin gained 0.9 gm., as did those in Group 20 (Table 3) receiving 5 mg. of MH, 0.2 mg. of prednisolone, 1 mg. of STH, and 5 μg. of thyroxin.

The MH used did not elevate the rate of O_2 consumption from the hypophysectomized control level (20 cal./m.^{2}/hr.), whereas the combination of prednisolone and thyroxin used returned it to 35 as compared to 25 for prednisolone alone, and 30 for normal females of this age.[3]

DISCUSSION

The results obtained in these experiments confirm the earlier findings that MH is "lactogenic"—which simply means that it is active in milk production. The claim that this hormone is a potent factor in initiating milk secretion has never been seriously questioned. But with the introduction of a new use for the term "galactopoietic," namely, as a label for hormones that enhance or maintain lactation already in process, the role of MH, here, has been less clear, especially when judged by its effect in dairy animals and women. In this attempt to quantitate MH's activity as a "continuing lactogenic" or galactopoietic factor, it would appear that it is as necessary in maintaining lactation in the hypophysectomized rat as in initiating it. And the necessity of having the mammary gland under the influence of an adrenocortical hormone seems to be equally important in the maintenance and induction of milk secretion.

From the data collected it may be said that MH and cortisol need not be supplemented with STH nor thyroid hormone in bringing a hypophysectomized rat's mammary glands into substantial secretory activity and maintaining them at a functional level considered to be about 50% of normal. Even at the subnormal growth rate observed (approximately 1 gm. per day as compared to the normal increment of about 2) young could supported and weaned with a body weight of about 30 gm. by hypophysectomized mothers injected only with MH and cortisol. In these experiments the weight increment in the young was counterbalanced by a decrement in the mother. Possibly this loss might not be tolerated for much longer than the three-week maximum followed in a few of these experiments; and a tissue-restoring hormone such as STH might indeed be important for long sustained lactations.

In searching for explanations for STH not bettering the milk production appreciably—if at all—one meets the possiblity that some tissues in a rat deprived of its pituitary for two weeks develop a greater need for, and have a higher priority for STH than has the mammary gland. In one

[3] Through the courtesy of Dr. Edward S. Evans of the Department of Anatomy, University of California.

experiment in which the high dose of 4 mg. of STH per day was added to the MH and cortisol, the mothers showed an average daily body weight gain of 4 gm., but the milk production decreased.

In Cowie's (5) experiments on substitution therapy in rats hypophysectomized on the 4th day of lactation, none of his combinations of hormones (even MH, STH, ACTH, thyroxin plus oxytocin) maintained milk production at more than 50% of normal. His best results were obtained in two rats that received MH and ACTH. These were consistent with our findings in rats injected with MH and cortisol. He noted some maintenance of lactation with prolactin alone (a commercial preparation containing 20–25 I.U. per mg. and less than 0.5% ACTH).

An unknown factor in Cowie's experiments, and in ours, was the oxytocin titre. His experiments were performed immediately after interruption of the oxytocin pathways and he injected this hormone two–three times daily in 0.5–0.7 I.U. doses. This was necessary because of a temporary deficiency but not a complete absence of a supply of endogenous ocytocin (22), (23), (24). It is noteworthy that previously Benson and Cowie (24) had reported that posterior-lobectomized rats given 0.75 I.U. of oxytocin two or three times daily did not show better than 50% of normal milk production. In their case it could not be assumed that the anterior lobe was functioning normally; and in ours, it probably should be assumed that the mechanism for the release of oxytocin in such a way as to ensure normal rhythmic movements in the milk conduction system was defective. This need not imply that the correction of such a defect would suffice to correct our 50% milk output deficiency. For that it seems more likely that multiple small corrections and substitutions will have to be made particularly in the way of carefully balanced amounts of the so-called metabolic hormones such as thyroxin, cortisone, aldosterone, insulin, STH, etc.

Since lactation is a most complex phenomenon involving and often necessitating the actions of many substances, such as hormones, vitamins and enzymes, it follows that animals will exhibit degrees of lactation reflecting the adequacy of each important factor. It is, therefore, difficult to set up a standard of milk secretion and delivery to be known as "basic lactation." Average norms for intact rats fed a supposedly complete diet have been established. Perhaps only these should be considered basic, and anything less, subnormal. In that event, it can only be claimed that the experiments described here have indicated a very simple, abbreviated formula of two hormones (MH and cortisol) that restores 50% of a total lactational deficit in rats deprived of their pituitaries.

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THE LOCALIZATION OF I^{131}-LABELED TRIIODOTHYRONINE AND THYROXINE IN THE PITUITARY AND BRAIN OF THE MALE GUINEA PIG[1]

DONALD H. FORD AND JACK GROSS

ABSTRACT

The brains and pituitaries of guinea pigs injected with I^{131}-labeled triiodothyronine showed an active concentration of radioactive material identifiable as triiodothyronine. In the brain the labeled hormone was found by autoradiographic procedures to be primarily in the nuclear areas, with the greatest accumulations occurring in the neurosecretory regions. The anterior pituitary lobe was consistently more active in concentrating the injected material than was the posterior lobe.

When I^{131}-labeled thyroxine was injected, the brain and both pituitary lobes were able to concentrate only very small amounts of the labeled hormone, the anterior lobe appearing to concentrate the hormone as readily as the posterior lobe.

IT HAS been shown in recent studies of the rabbit (1, 2) that triiodothyronine, as well as thyroxine, concentrates in the posterior pituitary. In addition, the adenohypophysis and the gray matter of nervous tissue were also observed to be capable of concentrating the labeled thyroid hormone, but to a lesser degree. The adenohypophysis was far more active than the nervous tissue in this respect. The neural gray matter concentrated the radioactive material about twice as effectively as did the white matter. The region of the hypothalamus was observed to take up more of the thyroidal hormone than did other neural areas. The median eminence and paraventricular region showed quite striking autoradiographic localizations which were of the same order of intensity as those observed autoradiographically in the posterior pituitary.

Although the uptake of labeled thyroxine by the pituitary and brain has been studied in species other than the rabbit, information relative to the uptake of triiodothyronine is lacking. Therefore, the uptake of triiodothyronine by neural and pituitary tissue in various other species has been studied. The present report describes our results of the uptake of triiodothyronine by neural and pituitary tissue in the guinea pig.

MATERIALS AND METHODS

The I^{131}-labeled triiodothyronine used in these experiments was obtained from the Oak Ridge laboratory of the Abbott Corporation, in a 50% propylene glycol solution.

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The material was checked for purity chromatographically and found to contain at least 82% triiodothyronine. Dilutions with normal saline were prepared in siliconized glassware to enable us to inject the proper dose intravenously into each animal in a volume of from 0.5 to 1.0 ml.

Male guinea pigs which weighed from 600 to 800 gm. were used. In the first group, consisting of 20 animals, the total radioactivity in various parts of the formalin-fixed brain and pituitary was determined by counting with a scintillation counter. From these data, the per cent of the dose of injected triiodothyronine present in each tissue and the organ plasma ratios were determined. The animals within this first group were subdivided so that two animals were sacrificed ¼ hour after the injection of the radioactive hormone, while the remaining animals were split equally into six groups. These groups were sacrificed ½, 1, 2, 3, 4 or 7 hours after the intravenous hormone injection. The dosage used was 0.5 μg. of triiodothyronine/kg. (15.2 μc./kg.; range 3.1–28.3 μc. The great variation in μc. dosage is due to the differences in specific activity between various shipments of triiodothyronine received from Oak Ridge).

In the second group of experiments two animals were injected with 5.0 μg. of triiodo-

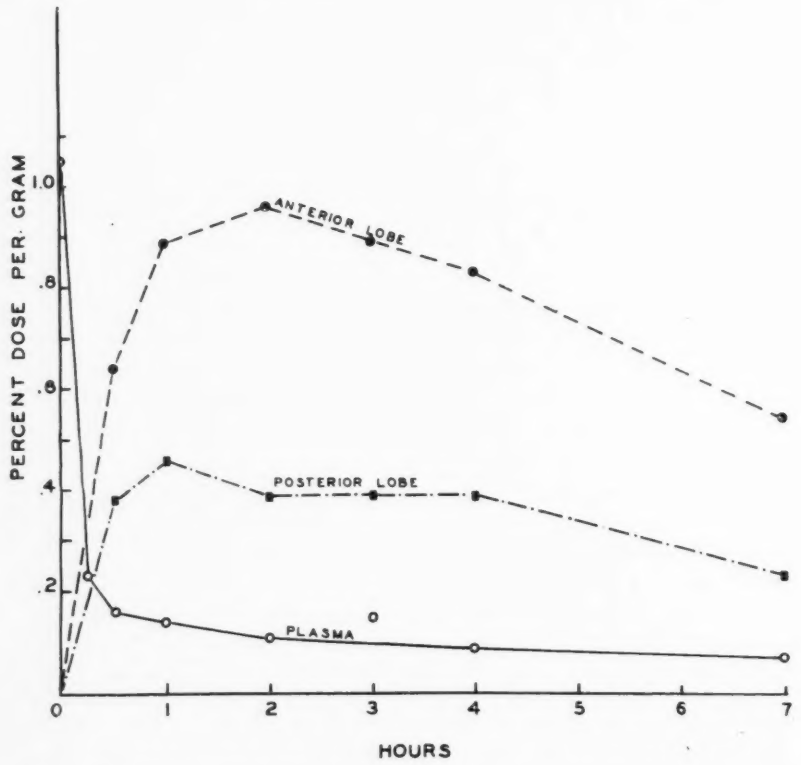

FIG. 1. The concentration of I^{131} in the pituitary lobes and plasma of guinea pigs receiving an 0.5 μg./kg. dose of I^{131}-labeled triiodothyronine intravenously. The individual points on the curves represent the mean values obtained from 3 animals, except for the first ¼ hour time, when only 2 animals were studied.

thyronine per kg. (100 and 114 μc./kg. respectively), and sacrificed 1½ and 2 hours later. The plasma and homogenized fresh tissues were studied chromatographically. In the third animal a dose of 18.0 μg./kg. (1 mc./kg.) was administered in 4 aliquots over a 45 minute period and the guinea pig was killed two hours after the last injection.

In the last group the autoradiographic localization of I^{131}-labeled triiodothyronine and thyroxine was determined. One animal was injected with iodide (I^{131}), three with thyroxine, five animals received triiodothyronine and one animal was an uninjected control. Those treated with triiodothyronine, thyroxine or iodide received a dose of 1 mc./kg. The hormone levels were from 38 to 43 μg./kg. for thyroxine and from 22 to 53 μg./kg. for triiodothyronine. The manner of injection was the same as for the animals which received the 18 μg./kg. dose in the chromatographic series.

Other specific details of the procedures used in these three types of experiments have been previously described (2, 3).

RESULTS

The concentration of I^{131} in the guinea pig plasma dropped sharply within the first ¼ to ½ hour after injection and a slower rate of fall was observed during the remaining six and a half hours of observation (Fig. 1). Chromatographic analysis of the plasma indicates that as the total plasma radioactivity fell the radioactivity attributable to the triiodothyronine decreased while the free iodide component increased (Table 1).

TABLE 1. CHROMATOGRAPHIC ANALYSIS OF THE METABOLITES OF I^{131}-LABELED TRIIODOTHYRONINE PRESENT IN THE PITUITARY, BRAIN AND PLASMA OF THE GUINEA PIG AFTER THE INTRAVENOUS INJECTION OF 5.0 μG./KG. OF THE LABELLED HORMONE*

	1 min.	60 min.	90 min.	2 hours
Plasma				
Triiodothyronine	86	69	61	56
Iodide	3	15	15	25
Residue	11	16	24	19
Anterior Pituitary				
Triiodothyronine			84	89
Iodide			3	3
Residue			13	8
Posterior Pituitary				
Triiodothyronine			66	76
Iodide			3	5
Residue			31	19
Mamillary Body				
Triiodothyronine			70	68
Iodide			11	6
Residue			19	24
Tuber Cinereum				
Triiodothyronine			67	71
Iodide			9	7
Residue			24	22
Cerebral Cortex				
Triiodothyronine			50	58
Iodide			18	6
Residue			32	26

* The figures represent the mean values from 2 animals.

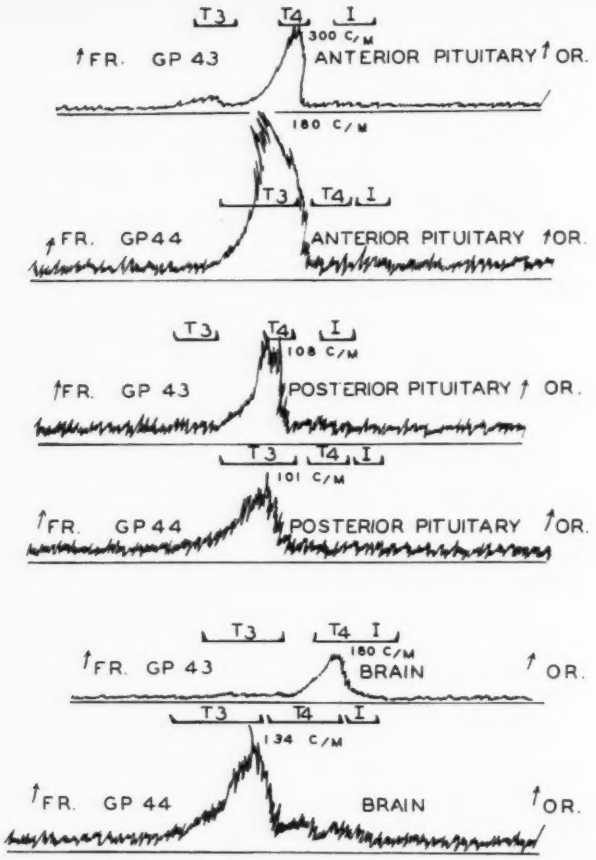

FIG. 2. An exact copy of scans of a radiochromatogram of the brain and pituitary lobes of guinea pigs (G.P.) injected with either I^{131}-labeled thyroxine (T_4) or triiodothyronine (T_3). Guinea pig 43 received 38 μg. of thyroxine/kg. while guinea pig 44 received 18 μg. of triiodothyronine/kg. (The dose in millicuries is 1 mc./kg.) OR = Origin; FR = Front; I = Iodide.

As the concentration of radioactivity fell there was an increase in the total radioactivity in the anterior and posterior pituitary lobes, which was highest in the adenohypophysis. The time of peak concentration was between 1 and 2 hours for both lobes (Fig. 1). The material responsible for the radioactivity in the pituitary lobes was predominantly triiodothyronine (Fig. 2, G.P. 44 and Table 1). The higher uptake by the anterior pituitary was consistently seen and is contrary to data reported for the rabbit (1, 2), where the posterior pituitary lobe concentrated more of the radioactive triiodothyronine. It may be noted in Table 1 that there was a slight increase in the radioactivity due to triiodothyronine in these tissues between 90 and 120 minutes.

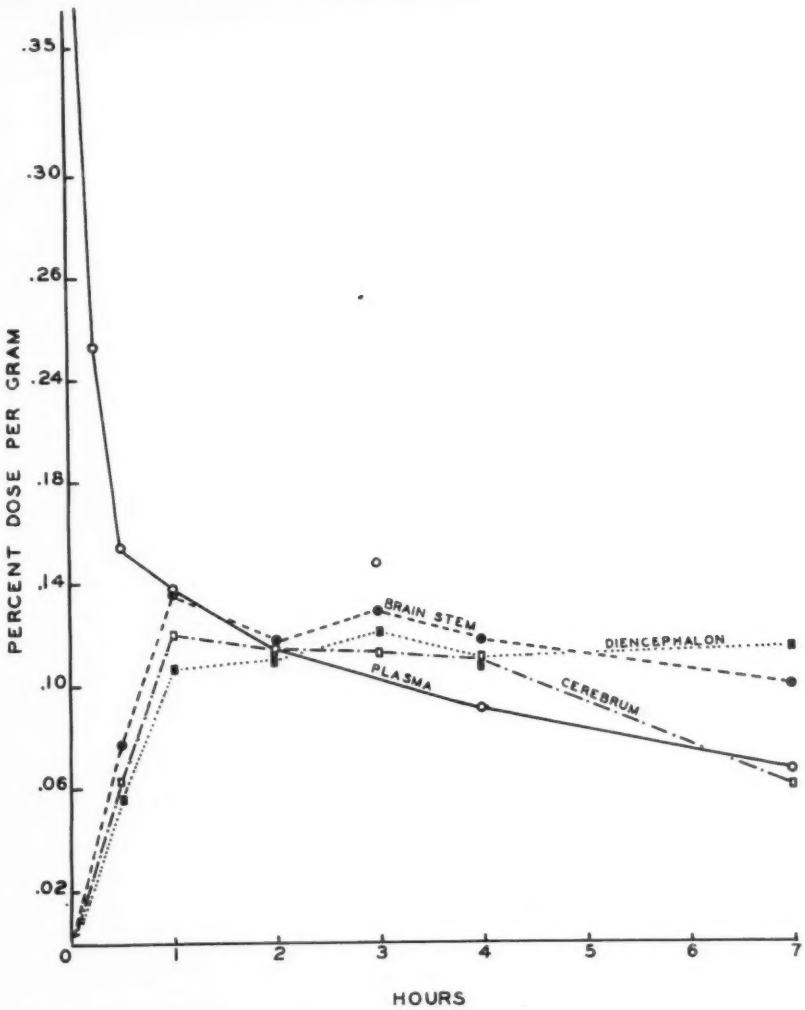

FIG. 3. The concentration of I^{131} in the brain stem, diencephalon, cerebrum and plasma of guinea pigs after an intravenous injection of 0.5 μg./kg. of I^{131}-labeled triiodothyronine. The individual points for the neural tissues represent the mean values from 3 animals.

The concentrations of radioactivity in the various parts of the guinea pig brain following injection of labeled triiodothyronine are shown in Figure 3. All the tissues studied showed a sharply rising uptake of activity during the first hour, followed by a plateau for at least 6 hours. Thus, while the uptake of I^{131} in the brain was not high, it maintained a positive gradient in comparison with the plasma levels. The only exception was the cerebral hemisphere, where the level of activity fell below the plasma level

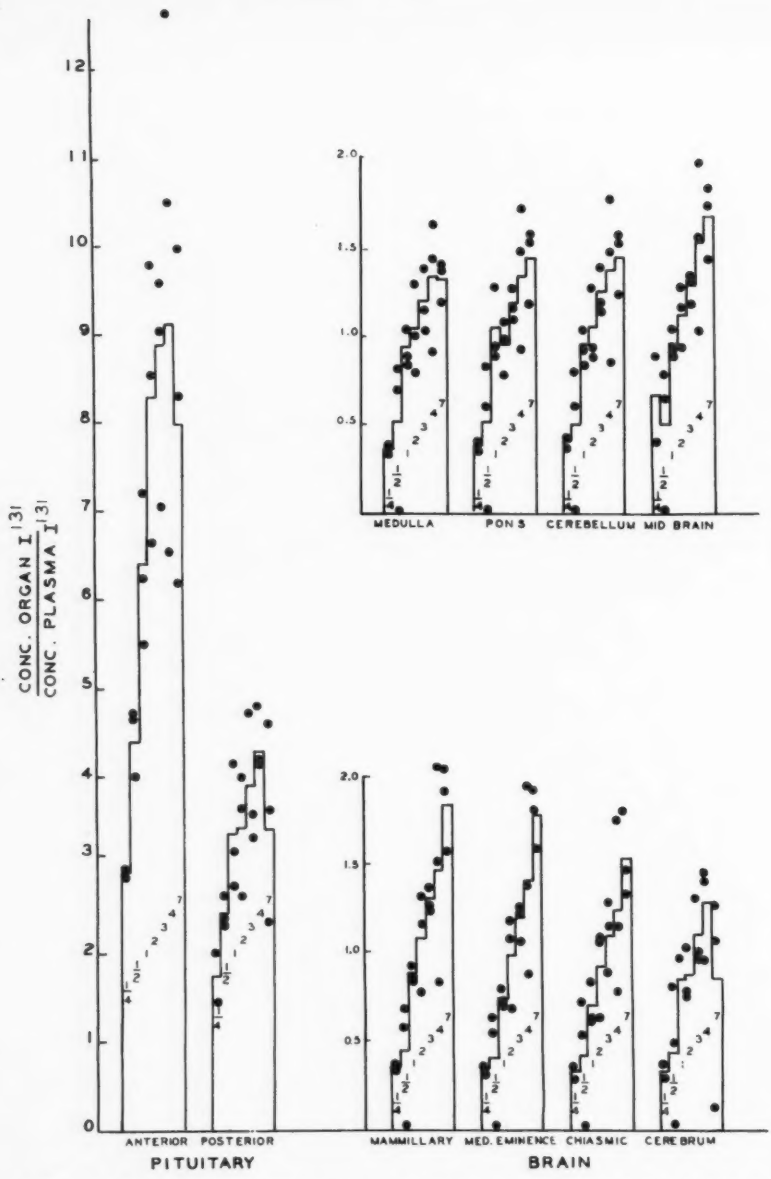

FIG. 4. Organ/plasma ratios of I^{131} in guinea pigs injected with I^{131}-labeled triiodothyronine, showing the change with time. The solid dots indicate the individual values.

seven hours after the injection. The brain radioactivity was due to the presence of the injected hormone (Table 1 and Fig. 2; G.P. 44). The per-

centage of the activity attributable to triiodothyronine increased with time in the neural tissues, despite falling levels of plasma triiodothyronine (Table 1). Additional evidence for an uptake of the thyroid hormone by the nervous tissue may be seen in Figure 4 where the organ/plasma ratios are plotted against time. Of the areas studied, the mammillary region and the median eminence appeared to retain the radioactive hormone the most readily.

Autoradiographs of sections of brain from iodide-injected or non-injected animals are not shown since the results were completely negative. The autoradiographic series of the brains from two well-perfused animals shown in Figures 5, 6 and 7 is illustrative of the results obtained from the other animals studied by this method. In these figures a comparison is made between the site and degree of localization of labelled triiodothyronine and thyroxine. There is little doubt but that more triiodothyronine was taken up by the brain and pituitary than was an equal dose of thyroxine. Comparable results were obtained from counting data in which the uptake of either triiodothyronine or thyroxine in the rabbit was compared (2). Furthermore, the predominance of nuclear concentration of radioactive material was clearer after triiodothyronine, probably owing to the greater concentration. The chiasmic diencephalon (Fig. 7) showed a particularly strong concentration in the supraoptico-paraventricular region in the triiodothyronine-treated animal. The region of the median eminence of the thyroxine-treated animal was the only area in which any appreciable autoradiographic reaction occurred. However, this concentration too was lower

KEY TO THE ABBREVIATIONS IN FIGURES 5, 6 AND 7

A.L.	Anterior pituitary lobe
C.	Caudate nucleus
D.H.	Dorsal horn of spinal cord
D.Sp.C.	Dorsal spinocerebellar tract
G.M.	Gray matter
I.C.	Internal capsule
I.O.	Inferior olivary nucleus
M.E.	Median eminence
M.L.	Medial lemniscus
N.C.	Nucleus cuneatus
O.C.	Optic chiasma
O.T.	Optic tract
P.L.	Posterior pituitary lobe
PY.	Pyramid
S.O.-P.V.	Supraoptic-paraventricular nuclear area
Sp.V.	Spinal tract of the trigeminal nerve
S.T.	Stalk of the pituitary
V.	Vestibular nuclear area
V.H.	Ventral horn of spinal cord
W.M.	White matter
XII	Hypoglossal nucleus

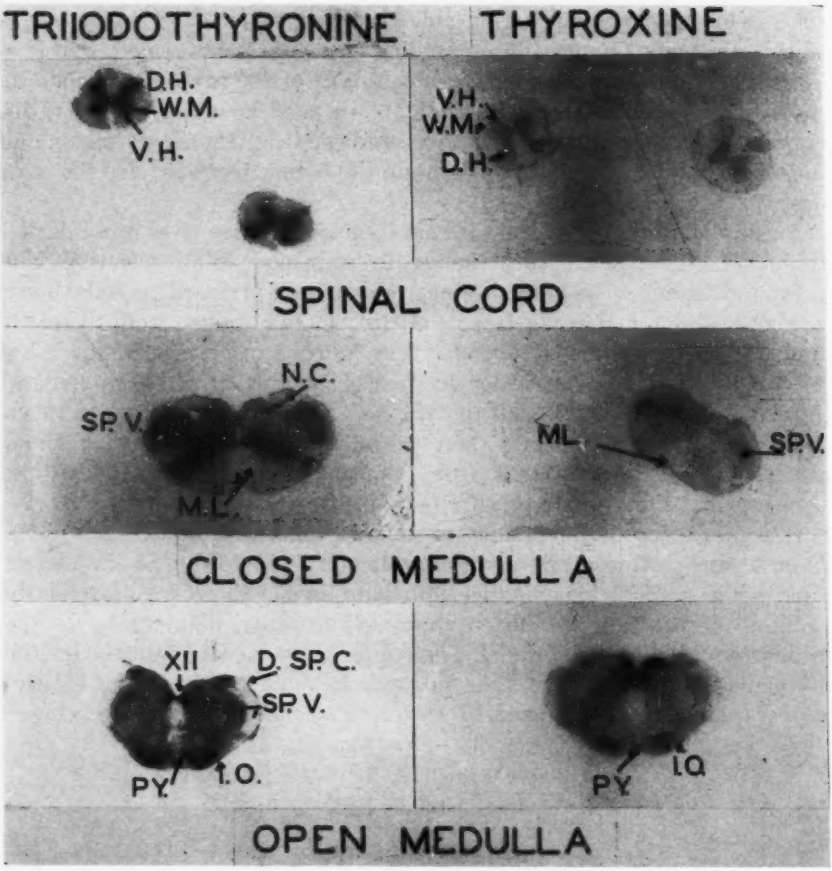

FIG. 5. Autoradiographic comparison of the localization of I^{131} in the spinal cord closed and open medulla of animals injected with either I^{131}-labeled thyroxine or triiodothyronine. The dosages were 1.0 mc./kg.

than that observed in many of the other nuclear areas of the triiodothyronine-treated animal (Fig. 7). The only other distinct accumulations of radioactivity in the thyroxine-treated animal appeared to be in the region of the inferior olive and the hypoglossal nucleus. Generally speaking, the localization in the thyroxine-treated animal was weak and according to nuclear groups.

While the degree of concentration of triiodothyronine in the anterior pituitary lobe was greater than in the posterior lobe (Fig. 7), it is of interest to note that thyroxine was concentrated almost equally in both pituitary lobes of the guinea pig, the anterior lobe appearing perhaps slightly more reactive than the posterior lobe in this respect. Thus, not

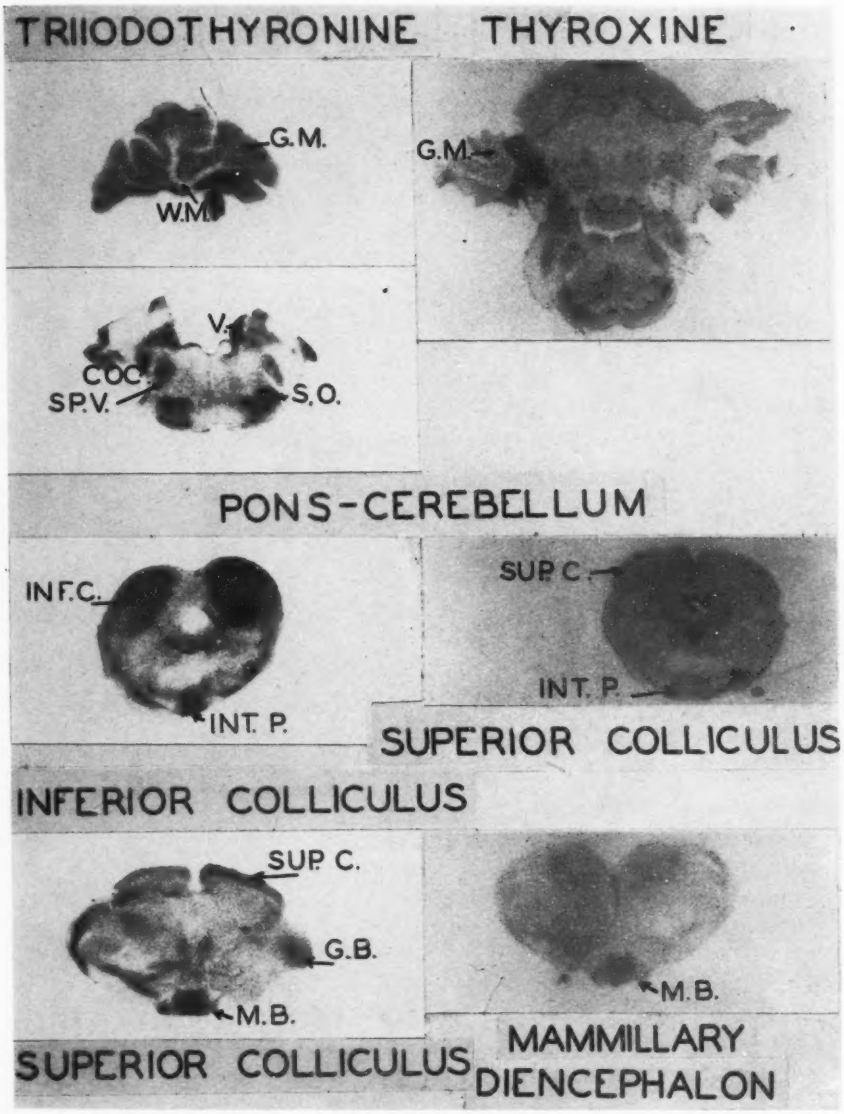

FIG. 6. Autoradiographic comparison of the localization of I^{131} in the pons, cerebellum, inferior colliculus, superior colliculus and mammillary bodies of animals injected with either I^{131}-labeled thyroxine or triiodothyronine. The dosages were 1.0 mc./kg.

only is the site of maximal triiodothyronine concentration in the pituitary lobes reversed from that seen in the rabbit, but the anterior lobe shows itself capable of concentrating thyroxine as well. As indicated in Figure 2,

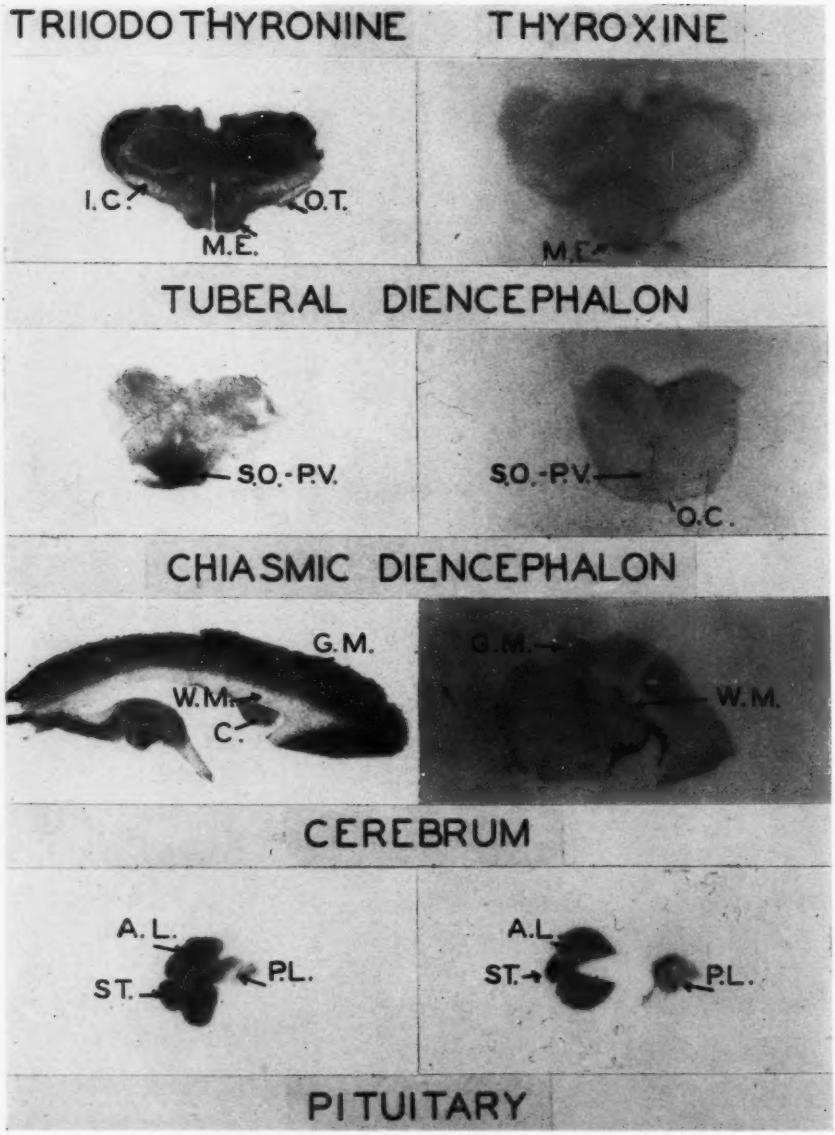

FIG. 7. Autoradiographic comparison of the localization of I^{131} in the tuberal diencephalon, chiasmic diencephalon, cerebrum and pituitary of animals injected with either I^{131}-labelled thyroxine or triiodothyronine. The dosages were 1.0 mc./kg.

the radioactivity responsible for these autoradiographic pictures was predominantly due to the presence of the injected labeled hormone. The autoradiographic studies also showed that the pars intermedia was only

capable of concentrating very small quantities of labeled triiodothyronine (Fig. 7).

DISCUSSION

The evidence presented shows clearly that an intravenous injection of I^{131}-labeled triiodothyronine is concentrated in the brain and pituitary lobes of the guinea pig. The radioactivity localized in these structures was found, by chromatographic analysis, to be primarily triiodothyronine.

Earlier studies (4) have indicated that the region of the CNS in which the most iodide could be found was the hypothalamus and midbrain. The present study, utilizing radioisotopic methods, demonstrates conclusively the hypothalamic concentration of iodide, after triiodothyronine injection which chromatographic analysis has shown to be primarily in the form of the injected labeled hormone (Fig. 2). This concentration of radioactivity was much less marked after an injection of thyroxine. Furthermore, the sites of maximal localization are primarily within the neurosecretory structures and secondarily the mammillary body (Fig. 4, 6 and 7) after triiodothyronine injection. Other nuclear areas also show a marked concentration. In addition, it is clear that the brain takes up more of the triiodinated form of the thyroid hormone than it does of the tetraiodinated form (Figs. 5, 6 and 7) within the two hour period in which peak concentration occurs.

While its significance still remains to be ascertained, the concentration of labeled hormone (triiodothyronine) in the supraoptic-paraventricular neurosecretory areas in both the guinea pig and the rabbit is most interesting (Figs. 7 and 4 [2]). This rather specific localization suggests that the hypothalamic concentrations of thyroid hormone (5, 6, 7, 8) may play some role in the normal functions of these nuclei rather than representing a concentration due to a hypothalamic filtering action which might aid it in controlling the rate at which the thyroid hormones reach the adenohypophysis (9). The observation that the anterior pituitary is able to concentrate the labeled hormone also makes such a filtering mechanism for the hypothalamus seem unlikely. Furthermore, hypothyroidism has been shown to stimulate neurosecretory function (10), while thyroxine inhibited it, again suggesting that the hypothalamic concentration of thyroid hormone may not be due to its ability to act as a filter between the blood borne hormone and the adenohypophysis. Von Euler postulated (11) that any neural control mediated by the hypothalamus on the function of the adenohypophysis might be through the neurosecretory hormones, whose release or retention might perhaps in this instance be dependent upon the level of thyroid hormone in the blood reaching the neurosecretory centers. He inferred that these hormones might act upon the trophic cells of the adenohypophysis in such a way that their ability to elaborate their respective pituitary hormones would be enhanced, although it was still

presumed that their secretory activation depends upon the concentration of the circulating peripheral hormones which reaches them. Thus, the rather marked concentration of radioactivity seen in the region of the neurosecretory center may be related to some form of hypothalamic control over anterior pituitary function; acting, not as a filter, but as a mechanism which can facilitate the output of TSH.

A comparison of the guinea pig brain with the rabbit brain (2) shows that the per cent of concentration of the injected dose in the guinea pig was greater than that observed in the rabbit, as were the organ plasma ratios. The difference in the peak organ plasma ratio of 0.6 in the rabbit (2) and 1.5 in the guinea pig (Figs. 3 and 4) suggests that the rate of utilization or metabolic degradation of triiodothyronine may be slower in the guinea pig than in the rabbit. This concurs with the prevalent view that the guinea pig exists in what might be termed a hypothyroid state.

Analysis of the pituitary lobes shows that the anterior pituitary is more active in concentrating the injected radioactive hormone than is the posterior lobe (Figs. 1 and 7). In this, the guinea pig differs from the rabbit, where the posterior lobe is the more reactive. In addition, the region of the stalk actively concentrated both triiodothyronine and thyroxine, while the pars intermedia appears to concentrate very little of either of the two thyroid hormones. Furthermore, the guinea pig adenohypophysis was shown by autoradiography to concentrate thyroxine as well as triiodothyronine (Fig. 7). However, it should be pointed out that a part of this autoradiographic response may have been due to the formation of triiodothyronine within the anterior pituitary lobe (Fig. 2). An explanation of this triiodothyronine formation has been previously discussed for the rabbit (2) and the guinea pig (3).

Acknowledgment

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THE EFFECTS OF SEVERAL STEROIDS ON FECUNDITY IN FEMALE RATS

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ABSTRACT

Female rats were treated with steroids daily from 5 days before to 30 days after placing with males. At the higher dose levels no pregnancies were observed during the treatment period. On a weight basis, estrone and Vallestril were the most potent; norethynodrel and testosterone propionate were somewhat less active. Progesterone was least effective of the compounds studied. Nilevar, which has both anabolic and progestational activities, was nearly as effective as testosterone propionate in preventing pregnancy. 17α-(1-methallyl)- and 17α-(2-methallyl)-19-nortestosterone, both of which are potent progestins in the Clauberg assay, were also more effective than progesterone in preventing pregnancy in rats.

ALTHOUGH much has been written on the roles of the sex steroids in initiation and maintenance of pregnancy, their actions in inhibiting fecundity are less well defined. Selye *et al.* (1) reported that treatment of normal female rats with 4 mg. of progesterone daily resulted in ovarian atrophy and pituitary hypertrophy. Within 2 days after treatment started, estrous cycles ceased and the animals remained in diestrus. Lahr and Riddle (2) studied the problem further and found that 0.03 mg. did not alter the estrous cycle but 0.3 mg. slightly prolonged the diestrous phase. Phillips (3), however, found the critical dose to be 1.5 mg., lower doses being ineffective. In at least one case he observed an estrous smear on the day after an injection of 4 mg. On the other hand, Everett (4) has shown that in the constant-estrous rat, progesterone induces ovulation and corpus luteum development. It is also well established that estrogens cause an inhibition of pituitary gonadotropin secretion and resultant ovarian atrophy (5). Pretreatment with estrogens results in decreased breeding efficiency in rats (6) while treatment after ovulation prevents nidation and interrupts pregnancy in guinea pigs (7), cats (8), and rabbits (9). The effects of androgens have been reviewed by Dorfman and Shipley (10).

The present investigation was designed to compare the effects of some of the common steroid hormones with those of some of the newer synthetic steroids which show separation or combinations of hormonal activities.

MATERIALS AND METHODS

Adult female rats, about 3 months of age, were treated daily for 35 days, with subcutaneous injections of various steroids in oil. On and after the day of the 5th injection,

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TABLE 1. EFFECT OF VARIOUS STEROIDS ON FERTILE MATINGS. PERIOD IN WHICH FIRST FERTILE MATING OCCURRED IN FEMALE RATS TREATED DAILY FOR 5 DAYS BEFORE AND 30 DAYS AFTER PLACING WITH MALES

Compound	Daily dose, mg./kg.	No. of rats	First fertile matings				Not fertile
			During treatment	Days after treatment			
				1–30	31–60	61–150	
			%	%	%	%	%
Oil	—	100	91	9	—	—	—
Estrone	0.005	10	60	20	10	10	—
	0.01	7	0	71	0	29	—
	0.02	10	0	90	10	—	—
	0.05	10	0	80	10	—	10
	0.1	17	0	35	6	12	47
Testosterone propionate	0.1	9	78	22	—	—	—
	0.2	10	10	80	10	—	—
	0.5	9	0	67	22	—	11
	1.0	8	0	63	37	—	—
Progesterone	0.2	10	70	30	—	—	—
	1.0	10	60	40	—	—	—
	2.0	10	20	70	—	10	—
	5.0	10	0	0	40	40	20
Vallestril	0.005	10	30	40	30	—	—
	0.01	8	0	100	—	—	—
	0.02	7	0	56	44	—	—
	0.05	4	0	50	25	25	—
	0.1	9	0	33	44	—	22
Norethynodrel	0.1	10	80	20	—	—	—
	0.2	10	0	90	—	—	10
	0.5	20	0	100	—	—	—
	5.0	10	0	0	30	20	50
Nilevar	0.005	10	70	30	—	—	—
	0.1	20	50	45	5	—	—
	0.2	19	42	37	11	—	—
	1.0	10	0	80	10	—	10
	5.0	8	0	50	25	13	12
SC-4956	1.0	10	60	30	10	—	—
	5.0	11	64	36	—	—	—
SC-6583	5.0	10	0	70	20	10	—
SC-6924	0.1	10	100	—	—	—	—
	0.2	20	95	5	—	—	—
	0.5	20	70	10	5	15	—
	1.0	8	25	63	12	—	—
	10.0	5	0	80	—	20	—
SC-8117	0.2	10	90	10	—	—	—
	0.5	10	40	60	—	—	—
	1.0	9	11	89	—	—	—
	2.0	9	0	89	—	11	—
SC-9022	0.1	10	90	10	—	—	—
	0.2	10	90	10	—	—	—
	0.5	10	20	70	10	—	—
	1.0	10	0	90	10	—	—

each 2 females were housed with a normal, untreated male. The males were rotated at weekly intervals and cohabitation was maintained for 6 months or until the females were obviously pregnant.

Estrone, progesterone and testosterone propionate were selected as representative steroid hormones since these serve as standards in this laboratory. 17α-ethyl-19-nortestosterone, Nilevar, was selected since it is a potent anabolic agent with minimal androgenic effects (11) but does produce progestational reactions (12). 17α-ethynyl-17-hydroxy-5(10)-estren-3-one (Norethynodrel), has weak estrogenic activity and produces progestational responses under appropriate conditions (13). 3-(6-methoxy-2-naphthyl) 2,2-dimethylpentanoic acid (Vallestril), is purely estrogenic, but shows a relative separation of vaginal cornification and uterotropic potencies (14). 17α-(1-methallyl)-19-nortestosterone (SC-8117), and 17α-(2-methallyl)-19-nortestosterone (SC-9022), are potent progestins with little or no androgenic-anabolic action (12, 15). 17α-methyl-4,5-dihydro-19-nortestosterone (SC-6583), has weak progestational and anabolic potencies. 17α-methyl-estriol 3-methyl ether (SC-6924), is a weak estrogen but has marked effects on blood lipids (16). Diethylaminoethyl 0-methyl-podocarpate (SC-4596), was included since it is not estrogenic or androgenic, nor does it have progestational activity in the Clauberg test, but it does prevent ovulation in estrous rabbits (17).

RESULTS

In adequate dosage, androgens, estrogens and progestins are all effective in preventing the normal course of pregnancy in rats. Table 1 shows the period in which the first fertile mating occurred in female rats treated with various steroids. If inhibition of fertile matings during the treatment period is taken as the criterion of effectiveness, the relative activity of some of these synthetic steroids can be compared with "standards" in several bioassays.

In Table 2 the estrogenic activities of some of these are compared. For

TABLE 2. COMPARISON OF ESTROGENIC ACTIVITIES. RELATIVE POTENCIES IN VARIOUS ASSAYS

Compound	Rat vaginal smear	Mouse uterine weight assay	Inhibition of pregnancy
Estrone	100	100	100
Vallestril	100	20	100
SC-4642	4	7	5
SC-6924	0.8	1.6	1

these, the inhibition of fertile matings appears to correlate quite well with estrogenicity as measured in the rat vaginal-cornification assay.

Some of these steroids are weak androgens. These have been compared with testosterone propionate in Table 3. The pregnancy-inhibiting effects of Nilevar, SC-6583 and SC-8117 are greater than would be expected from their androgenic activity. This activity might better correlate with the anabolic potency. However, these compounds also show progestational activity and their anti-fertility potency may be related to the latter activity.

Progestational potencies by both intrauterine and subcutaneous assays

TABLE 3. COMPARISON OF ANDROGENIC, ANABOLIC AND ANTI-FERTILITY ACTIVITIES IN RATS. RELATIVE POTENCIES IN VARIOUS ASSAYS

Compound	Androgenic activity	Anabolic activity	Inhibition of pregnancy
Testosterone propionate	100	100	100
Nilevar	6	100	50
SC-6583	10	10–100	>10
SC-8117	1	5	20

are shown in Table 4. Norethynodrel shows a much greater antifertility effect than would be expected from its progestational action. This undoubtedly is due to its estrogenicity as was pointed out earlier. SC-8117 appears slightly less effective as an anti-fertility agent than might have been predicted from its progestational potency in the rabbit, and the same

TABLE 4. COMPARISON OF PROGESTERONE-LIKE ACTIVITIES AND INHIBITION OF PREGNANCY. RELATIVE POTENCIES IN VARIOUS ASSAYS

Compound	Intrauterine assay	Clauberg assay	Inhibition of pregnancy
Progesterone	100	100	100
Nilevar	25	500	500
Norethynodrel	<5	25	1000
SC-4956*	<5	<5	<40
SC-6583	<5	10	>40
SC-8117	1000	500	200
SC-9022	1000	2500	500

* Active as progesterone in inhibition ovulation in rabbit.

is true for SC-9022. On the other hand, the potency of SC-6583 correlates better with its androgenic-anabolic activity.

DISCUSSION

Estrogens and progestins act at different phases of the cycle. The progestins and androgens appear to inhibit estrus, and are anti-estrogenic in animals treated with estrone (18, 19). On the other hand, estrogens inhibit endometrial development and prevent nidation in some species (7, 8, 9). In this study estrogens have proved most potent as inhibitors of pregnancy.

The long period of treatment used here was arbitrarily chosen in order to provide a broader base for the study of the recovery phase. At minimal effective doses, recovery of fertility was quite rapid with all compounds except progesterone.

These rats were pretreated for 5 days in order to avoid the interference of any possible gonadotropin release in response to the steroid treatment. A study of the effects of a single injection of these steroids at various times of the cycle is the subject of a future communication.

High doses of estrogens are known to result in the inhibition of pituitary gonadotropin secretion and the doses that prevented pregnancy in the

present study were high. Werthessen, *et al.* (20) studied the effects of estrogen-progesterone combinations on rabbit blastocysts and found that while stilbestrol inhibited growth, estradiol at low dosage actually stimulated growth. In our series, at the higher doses, all of the estrogens inhibited pregnancy. Whether low doses would stimulate pituitary gonadotropin secretion could not be determined from our data, since pregnancy ensued quite promptly after cohabitation in our control series.

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THE EFFECTS OF SEVERAL STEROIDS ON MATING BEHAVIOR, OVULATION AND PREGNANCY IN FEMALE RATS

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ABSTRACT

Female rats were treated with a single injection of estrone, progesterone, norethynodrel or 17α-(2-methallyl)-19-nortestosterone (SC-9022) on various days from late diestrus to the 10th day of pregnancy. All animals were sacrificed on the 15th day after mating. Progesterone and SC-9022 administered in late diestrus or proestrus showed an anti-estrogenic action and the females refused the males. Administered after mating, these compounds had no effect. Estrone and norethynodrel administered on the day of mating or before nidation, prevented nidation in some or most cases. After nidation, norethynodrel treatment did not interrupt pregnancy.

IN AN earlier paper (1) the effectiveness of various sex hormones interfering with some steps in the series of events which leads to the production of offspring was reported. The present study was undertaken in an attempt to determine the mode of action of these diverse compounds.

MATERIAL AND METHODS

The steroids used were estrone, progesterone, norethynodrel (17α-ethynyl-17-hydroxy-5(10)-estren-3-one) and 17α-(2-methallyl)-19-nortestosterone (SC-9022).

Daily vaginal smears were taken from adult female rats. A single subcutaneous injection of the several steroids in oil was given on various days before or after mating. The animals were sacrificed on the 15th day after mating. The number and the size of the placentae were determined.

RESULTS

In the early phases of this work no record was kept when the female refused the male. It soon became evident, however, that females treated with progesterone or SC-9022 in late diestrus failed to progress into full estrus and refused to accept the male. Of 25 females treated with 5 mg. per kg. progesterone, only 1 mated. Of 25 females similarly treated with 0.5 mg. SC-9022 per kg., none mated. In general leucocytes were found in the vaginal smears of these animals on the day after injection. When the rats were treated with progesterone or SC-9022 on the day of proestrus, many failed to progress into full estrus. However, some did breed and these are included in the following tables.

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TABLE 1. EFFECT OF TREATMENT WITH VARIOUS STEROIDS ON OVULATION IN RATS

Compound	Dose, mg./kg.	Day*	No. of rats	% with placentae
Controls			42	98
Estrone	0.05	−1	9	89
		0	11	100
		1	11	27
Progesterone	5.0	−1	1	
		0	10	100
		1	10	100
Norethynodrel	0.5	−1	24	100
		0	23	91
		1	21	76
SC-9022	0.5	0	12	100
		1	10	90

* Day on which female rat was injected. Day 0 is the day of procstrus.

The effects of treatment on implantation are shown in Tables 1 and 2. The figures in these tables include all rats with placentae whether or not these placentae contained living fetuses at the time of necropsy. However, included in Figure 1 are only those placentae that contained normal appearing fetuses.

In most cases the larger placentae were associated with normal appearing fetuses. In some cases, however, small resorbing fetuses were found while in others resorption of the placenta was advanced and no fetal remnants were evident. Further information was obtained by weighing groups of 10 or more fetuses from several groups. In most groups, the average fetal weight was 175 to 185 mg. In two groups, less than 10 fetuses were available for study. In the group treated with estrone on day 1, the average weight was only 87 mg. for 7 and on day 2, 129 mg. for only 2 fetuses. There was some impression that the anterior portions of these smaller

TABLE 2. EFFECTS OF TREATMENT WITH VARIOUS STEROIDS ON IMPLANTATION IN RATS

Compound	Dose, mg./kg.	Day*	No. of rats	% with placentae
Controls	—	—	42	98
Estrone	0.05	2	11	9
		3	13	8
Progesterone	5.0	2	10	100
		3	10	100
Norethynodrel	0.5	2	20	100
		3	20	45
		4	20	75
		5	10	100
		6	10	100
		10	10	100
SC-9022	0.5	2	9	100
		3	10	100
		4	10	100

* Day on which female rats were treated. Day 1 is the day sperm were found in the vagina.

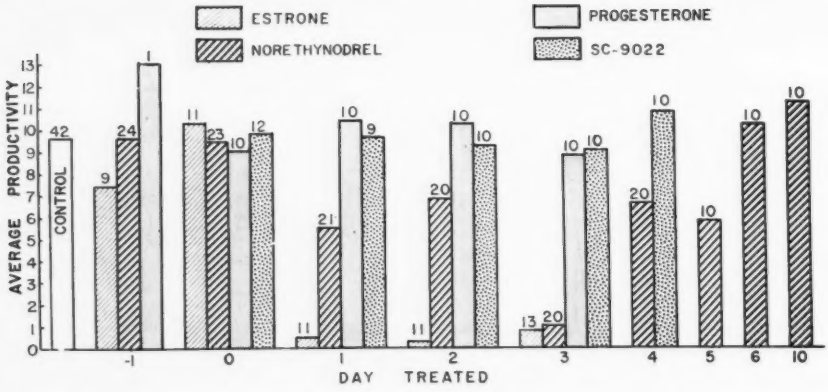

FIG. 1. Average productivity (total normal fetuses/number of rats bred) of female rats treated with a single injection of sex steroids on the day shown. Day 1 is the day sperm were found in the vagina. All rats were sacrificed on day 15 after breeding. The doses used were as follows: estrone, 0.05 mg. per kg.; progesterone, 5 mg. per kg.; norethynodrel, 0.5 mg. per kg.; SC-9022, 0.5 mg. per kg.

fetuses showed less complete development. It must be emphasized that these observations were on very limited numbers since most females in these groups had no normally developed fetuses. With progesterone treatment on day 0, the fetuses tended to be slightly heavier, 193 mg., but those injected on day 1 were in the normal range (176 mg.). In the norethynodrel-treated group, fetal weights were reduced only in the groups treated on days 2 and 3 (112 and 93 mg.).

DISCUSSION

The anti-estrogenic effects of progesterone have long been recognized. Similar activity has also been reported for SC-9022 (2). On the other hand, while norethynodrel exhibits progestational activity, it is also estrogenic (3). In this series norethynodrel did not interfere with acceptance of the male.

According to Long and Evans (4), in the rat the proestrous stage lasts 12 hours and is followed by 12 hours of estrus, during which the female accepts the male. Ovulation occurs toward the end of this period or within 20 hours after copulation. While it is possible that some of the inhibitory effects observed with estrone injected during the first day after mating were due to inhibition of ovulation or fertilization (5), other explanations appear more likely. Greenwald (6) has shown that treatment of rabbits with estradiol within 48 hrs. *post coitum* reduced the mucin layer surrounding the egg and interrupted pregnancy. Such effects could be obtained with small doses which did not interfere with endometrial development. When treatment was initiated later, pregnancy was interrupted only by larger doses of estradiol, which also interfered with the progestational response in the uterus.

Griffith and Farris (7) state that implantation occurs on day 5 or 6. Allen (8) showed that estrogens interfere with the endometrial response to progesterone in the rabbit. The dose used in our study was moderately large and would be expected to inhibit endometrial development. Whitney and Burdick (9) showed that large doses of estrone will lock the ova in the oviducts if injections are begun not later than the second day. However the doses required were so large that this mechanism would seem to be excluded in the present series.

The net effects of the various treatments on fecundity are illustrated in Figure 1. This depicts the average number of apparently normal fetuses in all rats that mated. Some of these females had resorbing placentae in their uteri but in other cases no evidence of implantation was found at time of necropsy. The graph does not depict the contraceptive potencies of progesterone or SC-9022 administered in late diestrus since females so treated usually refused the males. Since these steroids prevent pregnancy in rats (1) it would appear that they act primarily by inhibiting estrus. Over the longer term they may also be depressors of pituitary gonadotropin secretion and this point is currently under investigation. From these data, none of the compounds studied appear to act as inhibitors of ovulation *per se* in the rat. With continued administration they do cause gonadal atrophy and thus follicles fail to develop but this cannot account for the effect of a single injection in this series. The well established effect of estrogens in inhibiting the progestational response in the rabbit (8, 10) would appear to explain the anti-fertility effects observed with estrone and norethynodrel in the present series. Once implantation has occurred, this antiprogestational effect of estrogens is less important, as was also shown by Courrier and Gros in cats (11). In this study, norethynodrel, in the dose used, prevented nidation in some cases but did not interrupt pregnancy after nidation had occurred.

Acknowledgment

The author wishes to express his thanks to Mrs. Betsy Robb for her capable technical assistance.

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THE PARTITION AND BINDING OF CALCIUM IN THE SERUM OF THE LAYING HEN AND OF THE ESTROGENIZED ROOSTER[1]

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ABSTRACT

Total calcium of serum was increased in roosters from a normal value of 10 mg. per cent to 100 mg. per cent or higher at about 5 days following an injection of 125 mg. of estrogenic substance (Ayerst). With this tenfold increase in the total serum calcium, there was a *pari passu* appearance and increase in concentration of a phosphoprotein complex, found normally in the serum of the laying hen, but not found, under usual conditions, in the serum of the rooster. This included two specialized proteins, X_1 (a phosphoprotein) and X_2 (a phospholipid lipoprotein). Chylomicrons and beta-lipoproteins were increased and alpha lipoproteins were decreased in concentration at the same time. When the total calcium was elevated to 100 mg. per cent, beta lipoproteins appeared to bind approximately 10 mg. per cent; albumin 5 mg. per cent; and X_1 and X_2 (chiefly X_1) 79 mg. per cent. Only approximately 6 mg. per cent of the total serum calcium in both control and estrogenized roosters was present in ultrafilterable form. Since the ultrafilterable fraction of the serum calcium did not change during the rapid and dramatic rise of the total calcium, it follows that the added calcium was entirely in the nondiffusible fraction. Moreover, from the evidence of the ultracentrifuge, the major portion of the increase in non-filterable calcium was held in an undissociated complex with the phosphoprotein. There was no evidence for the presence of a colloidal calcium phosphate complex. Calculations have been made of the *capacity* of the mixed serum proteins of the estrogenized fowl for the binding of calcium, and these proteins were found to have a much greater capacity, per unit weight, for combining with calcium than any other protein, including casein, previously studied. On the other hand, when the dissociation constant of the calcium-protein complex was calculated, it did not appear to be markedly different from that of other proteins, indicating that the *affinity* for calcium was essentially the same. The phosphoprotein fraction was deposited in the granules of egg yolk, in the laying hens studied, with the full complement of calcium with which it was associated in the serum.

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THE hypercalcemia of laying hens and of estrogen-treated birds of both sexes is one of the most striking alterations in the mineral metabolism of vertebrate animals. With the aid of the ultracentrifuge it was recently observed that the increase in the level of serum calcium in estrogenized roosters depends upon the appearance in plasma of a highly specialized phosphoprotein complex (1). These experiments were done on serum diluted with 8.8% salt solution to remove the chylomicrons and beta lipoproteins by flotation, and to resolve the albumin and the denser globulins. The present experiments deal with the mechanism and measurement of calcium binding by the proteins individually and as a mixture in serum, and the circumstances surrounding the deposition of these specialized proteins in egg yolk.

MATERIALS AND METHODS

Twenty-five Black Minorca roosters were injected with either 100 mg. of crystalline estrone (U.S.P.) or 125 mg. of estrogenic substances (Ayerst). Ten untreated birds served as controls.

Blood from two or three birds of each group was pooled for each of the studies to be described below in a running account of the experiments. Representative figures for the composition of the serum of treated and untreated birds are shown in Table 1. The chylomicron fraction of the serum (containing most of the lipid and amounting to

TABLE 1. CHEMICAL COMPOSITION OF NORMAL AND ESTROGENIZED ROOSTER SERUM 5 DAYS AFTER INJECTION OF EITHER 100 MG. ESTRONE (USP) OR 125 MG. ESTROGENIC SUBSTANCES (AYERST)

Component*	Control rooster†			Estrogen-treated rooster
	A	B	C	
Total calcium, mg.%	10.00	8.00	12.6	97.00
Ultrafilterable calcium	6.00	5.00	6.2	6.00
Total protein, gm.%	4.00	3.90	5.5	7.40
Albumin, gm.%	1.00	1.00	1.00	0.60
Globulin, gm.%	3.00	2.90	4.50	6.80
A/G ratio	0.33	0.34	0.20	0.08
Inorganic phosphorus, mg.%		6.20		20.00
Total phosphorus, mg.%		16.50		189.00
Alkaline phosphatase, mg.%		11.50		7.10
Phospholipid, mg.%		191.00		4,264.00
Total lipid, mg.%		710.00		14,210.00
Blood sugar, mg.%		217.00		197.00
Citric acid, mg.%		23.90		8.90
Inorganic sulfate, mg.%		5.80		1.70

* The methods used for the above chemical determinations are as follows: *Calcium:* Clark, E. P. and J. B. Collip: *J. Biol. Chem.* **63:** 461. 1925. *Total protein:* Henry, R. J., C. Sobel and S. Berkman: *Anal. Chem.* In Press. *A/G ratio:* Wolfson, W. Q., C. Cohn, E. Calvary and F. Ichiba: *Am. J. Clin. Path.* **18:** 723. 1948. *Inorganic P:* Fiske, C. H. and Y. Subbarow: *J. Biol. Chem.* **66:** 375. 1925. *Total P:* Dryer, R. L., A. R. Tammes and J. I. Routh: *J. Biol. Chem.* **225:** 177. 1957. *Alk. phosphatase:* Shinowara, G. Y., L. M. Jones and H. L. Reinhart: *J. Biol. Chem.* **142:** 921. 1942. *Phospholipid:* Youngburg, G. E. and M. V. Youngburg: *J. Lab. Clin. Med.* **16:** 158. 1930. *Total lipids:* Gertler, M. M. and B. S. Oppenheimer: *Circulation.* **7:** 533. 1953. *Glucose:* Folin, O. and H. Wu: *J. Biol. Chem.* **41:** 367. 1920. *Citric acid:* Taussky, H. H. and E. Shorr: *J. Biol. Chem.* **169:** 103. 1947. *Inorganic sulfate:* Letonoff, T. V. and J. G. Reinhold: *J. Biol. Chem.* **114:** 147. 1936.

† A = theoretical (these exact figures have been observed in one rooster). B = observed low. C = observed high.

approximately 10% of the volume of the estrogenized serum) was separated unsalted by flotation from all other fractions by the use of the preparatory ultracentrifuge with a field of 70,000 G for 12 hours. After removal of the chylomicrons, the serum was separated into four fractions with the aid of the partition cells designed by Schjeide and Dickensen (2). A sample of 10 lambda of serum was removed from each of the four segments of the partition cell for paper electrophoresis (15 amperes for 12 hours; bromphenol stain). Aliquots of the contents of each segment were also diluted with an equal amount of 17.6% sodium chloride to resolve the individual protein components for ultracentrifugal analysis. The schlieren patterns were recorded during ultracentrifugation at 50,740 RPM at 64, 80 and 96 minutes. Calcium and other analyses were carried out on the contents of each segment according to methods listed in the footnote to Table 1. For comparison, an analysis was made of the principal cations and anions of the serum of laying hens and of egg yolk. Other observations made for specific purposes are described in the appropriate sections below.

RESULTS

Calcium.—The total calcium was found to be elevated above the level of that observed in control plasma in all four segments of the partition cell. However, segments I and II, constituting the top fractions, contained only a slightly greater concentration of calcium than is found in control serum. Segment III contained twice the normal level of calcium, but segment IV (the bottom fraction) contained 20 times more than the control rooster serums.

Diffusible Calcium.—The diffusible calcium, as determined by ultrafiltration of whole serum, was approximately 6.0 mg.% in both control and estrogenized serums. The bottom segment had to be diluted with serum from untreated roosters to measure the ultrafilterable calcium because this segment had the consistency of thick syrup and no fluid would pass from it through the collodion membrane. The bottom fraction, containing 249 mg.% calcium, was diluted with control rooster serum (containing 10 mg.% calcium) in proportions of 1 to 3 by volume, and the mixture produced an ultrafiltrate containing only 6.0 mg.% calcium, or the same as from normal serum.

Nitrogen.—The total nitrogen content of segment I was slightly higher than that of segment II. This was due to the presence of a small concentration of lipoproteins, which float into the top segment with prolonged centrifugation. The total nitrogen content of segment IV was 4 to 6 times greater than that of other levels. These observations verified the estimates of the relative amounts of the serum proteins based upon schlieren patterns, which reflect (by the area of the various peaks) the absolute amounts (concentrations) of each of the serum proteins. With the use of a factor of 6.3, grams of nitrogen were converted to grams of protein. A large part of the protein produced by estrogen treatment is rich in phospholipid, and this introduces an element of uncertainty into the use of this factor. Analyses of whole serum indicate that the protein content may be increased by as much as 3.0 grams per cent by the effects of estrogen.

Electrophoretic Patterns.—Each one of the four segments contained a different complement of electrophoretically resolved proteins. Segment I, or the top fraction, contained a large amount of beta-lipoprotein and no detectable alpha-lipoprotein. Segment II showed essentially the same pattern, except for the presence of a small amount of albumin. Material from segment III contained a larger amount of albumin. Segment IV showed a new fast-moving component and a slow-moving gamma globulin peak. The total of the areas of the various components was greater than those of proteins normally found in the serum of roosters.

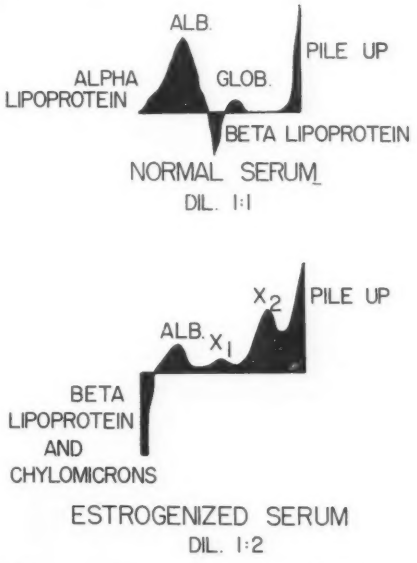
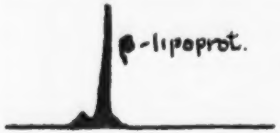
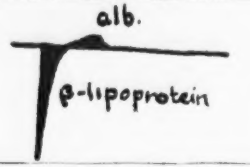
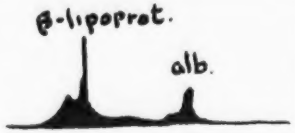
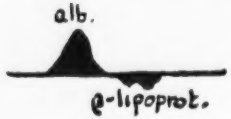
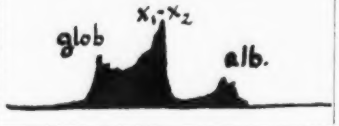
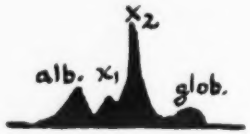


FIG. 1. Composite schlieren patterns of (above) the serum of a normal rooster, and (below) of a heavily estrogenized rooster. x_1 and x_2 are osmotically inactive new components secreted by the liver into the plasma in the form of complexes with calcium.

Ultracentrifugal Patterns.—The schlieren patterns corresponded very well with the electrophoretic patterns. The top fraction (segment I) showed a large peak which gave a negative index of refraction relative to the medium in which centrifugation was taking place. Such a negative refraction indicates the presence of a large amount of lipid and confirms the identification of the electrophoretic components in this fraction as beta-lipoproteins. A small amount of albumin appeared together with the beta-lipoproteins in segment II. Similar components appeared in segment III. In segment IV, or the bottom fraction, there were four distinct components characteristic of estrogenized avian serum: the fractions X_1 and X_2; albumin, and denser globulins. Conspicuously absent from this bottom fraction were both alpha-lipoprotein and beta-lipoprotein (Figs. 1, 2 and 3).

The Calcium-Protein Complex.—A list of serum proteins identified by their electrophoretic mobilities and sedimentation characteristics is shown in Figure 2, Column F. A rough estimate of the amount of calcium bound to each component is shown in Column G. These calculations are based

PARTITION OF CALCIUM IN ESTROGENIZED SERUM

Partition cell disc (A)	Mg.% Ca observed* (B)	Mg.% Total N (C)	Electrophoretic patterns† (D)	Ultracentrifugation patterns (E)	Proteins in each disc (F)	Grams per cent protein bound calcium‡ (G)
I	16.4	459.0			β-Lipo-Protein	10.4
II	18.0	386.0			β-Lipo-Protein	10.0
					Albumin	2.0
III	21.9	582.0			β-Lipo-Protein	10.9
					Albumin	2.0
					$X_1 - X_2$?	3.0
IV	258.0	2275.0			Albumin	8.0
					$X_1 - X_2$	240.0
					Globulin	4.0

* Total calcium was 97.0 mg.%. Ultrafilterable calcium was 6.0 mg.% on whole serum.
† Analatrol readings.
‡ Estimated according to the relative proportions of the height of the major density peaks and known calcium binding capacity in control rooster serum.

FIG. 2. Composite table and diagram showing the chemical, electrophoretic, and ultracentrifugal analyses of calcium and protein in estrogenized serum.

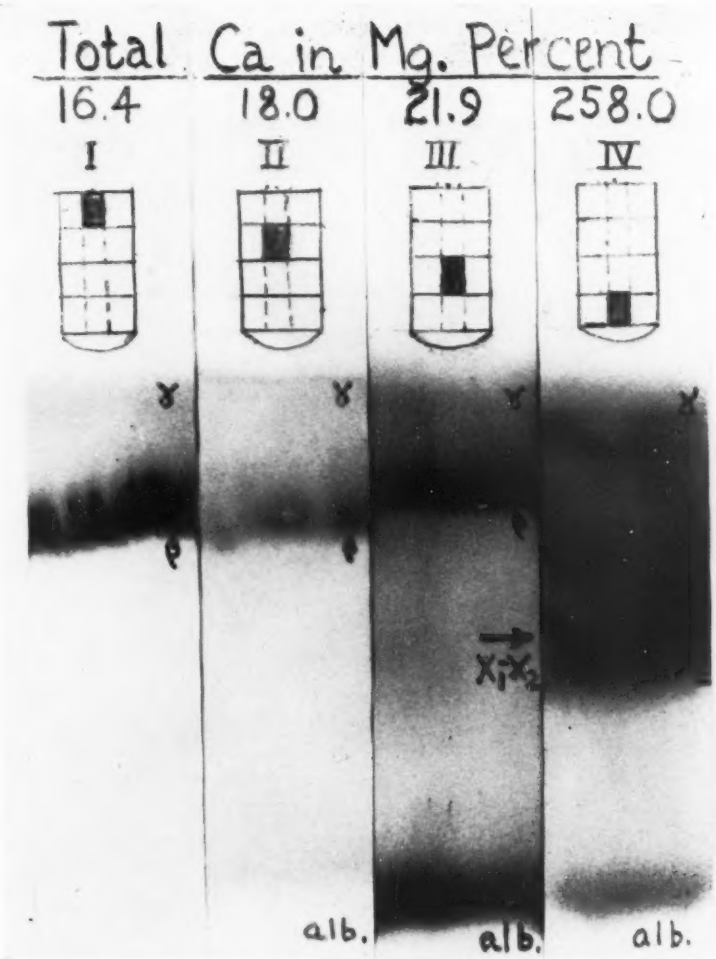


FIG. 3. Diagrams of the four segments of the partition cell used for separating the proteins in estrogenized serum. The calcium content of each segment is shown above the diagrams. The electrophoretic components of samples of serum removed from the same segments and indicated by the black rectangles, are shown below on paper stained with brom-phenol.

on the areas of the peaks, the amount of calcium in each segment of the partition cell and the amount of ultrafilterable calcium in whole serum. Relative to untreated rooster serum, beta-lipoprotein was increased in concentration. Albumin, the main calcium-binding protein in control rooster serum, was present in practically normal or in low concentrations. The A/G ratio was reduced from 0.34 in normal roosters to 0.08 in estro-

genized roosters. The protein chiefly responsible for binding the ten-fold increase in concentration of calcium was always located in the bottom of the partition cell, almost entirely in segment IV. This protein, specifically X_1, differed from other proteins in the serum, in that it contained a high percentage of protein phosphorus, in which respect it was similar to ovovitellin and casein.

The nature of the association between the calcium and the phosphoproteins in the serum was examined further with respect to the calcium-binding properties of these proteins. The direct approach to the isolation and purification of the two fractions, X_1 and X_2, separately or together, and their subsequent characterization, has so far failed. These proteins are insoluble in distilled water, and are kept in solution by the electrolytes of the serum. They are easily precipitated from the serum by dilution with distilled water, and presumably are separated from the normal protein constituents of the serum by this process. They may then be redissolved by the addition of salts of calcium and/or sodium, leaving only a minute risidue of insoluble material. When this is done, however, the proteins in the reconstituted solution have lost their electrophoretic mobility and a large part of their ability to combine with calcium, and are therefore denatured in these respects. This is in contrast with such proteins as serum albumin and globulin, and with casein, all of which are soluble in distilled water, and which do not undergo denaturation in such solutions. Moreover, dilution of the serum of estrogenized roosters with 0.9% sodium chloride solution also interferes irreversibly with the calcium-binding capacity of the phosphoproteins, as shown by the fact that the relationships between the diffusible and nondiffusible fractions of the serum calcium are not predictable following such dilution.

McLean and Hastings (3) found that the relationship of calcium and protein, in plasma or serum, could be described in terms of a solution of a weak electrolyte, calcium proteinate, the ionization of which is represented by the equation:

$$\mathrm{CaProt} \rightleftarrows \mathrm{Ca^{++}} + \mathrm{Prot^{=}} \tag{1}$$

and by the mass-law equation:

$$\frac{[\mathrm{Ca^{++}}] \times [\mathrm{Prot^{=}}]}{[\mathrm{CaProt}]} = \mathrm{K_{CaProt}} \tag{2}$$

which may be written in logarithmic form as:

$$\mathrm{pCa^{++}} + \mathrm{pProt^{=}} - \mathrm{pCaProt} = \mathrm{pK_{CaProt}} \tag{3}$$

in which p represents the negative logarithm. This formulation was extended to casein by McLean and Hastings, and has been repeatedly confirmed (4–9) as a first approximation of the calcium-protein relationship.

The formation of a complex of calcium with protein depends quantita-

tively upon three factors: 1) the concentration of Ca^{++} in the solution; 2) the *affinity* of the protein for calcium, which may be expressed as the dissociation constant, K_{CaProt}, or its reciprocal, the association constant; and 3) the *capacity* of the protein for combination with calcium, which may be expressed in terms of mols, which represent the number of calcium-binding groups per unit weight of protein, or correspondingly, the mols of calcium that would be combined with a unit weight of protein at infinite Ca^{++} concentration.

McLean and Hastings, in the absence of a satisfactory factor for converting grams of protein to mols of calcium-combining groups, made use of curves constructed from titration of protein solutions with alkali. This involved the assumption that carboxyl groups, neutralized by alkali, were available for combination with calcium. Greenberg, Larson and Tufts (7) avoided this assumption by writing the mass law-equation in the form:

$$\frac{\text{Protein (gm./100 cc.)}}{\text{CaProt (mg. Ca/100 cc.)}} = \frac{1}{A} + \frac{B}{Ca_D{}^{++}\text{(mg./100 cc.)}} \qquad (4)$$

in which A and B are constants. When Protein-CaProt is plotted against $1/Ca^{++}$ a straight lines is obtained (Fig. 4). The intercept of this line with

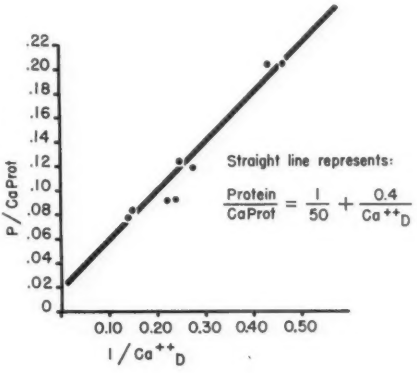


FIG. 4. Graph showing the relationship between the non-diffusible and the diffusible calcium of estrogenized serum diluted with partially decalcified serum from untreated control roosters. Constructed from data in Table 2.

the Protein/CaProt axis represents the reciprocal of the constant A, and the slope of the line gives the constant B. 1/A then gives a factor by which grams of protein must be multiplied to convert the protein to milligrams of calcium-combining capacity per gram protein. The product AB is the equilibrium constant for equation (4). Having obtained the factor, 1/A, the calcium-binding capacity of the protein may be substituted in equation (2) or (3), and the value K_{CaProt} or pK_{CaProt} obtained when Ca^{++} and CaProt are known and when the quantities in Equation (4) are calculated in molar terms. Klotz (8) and Carr (9) have made extensive use of this equation, and Carr has made calculations of the number of calcium-combining groups per molecule of protein. Carr (9) has also made direct estimates of

TABLE 2. DATA AND CALCULATIONS FROM AN EXPERIMENT IN WHICH POOLED SERUM FROM ESTROGENIZED ROOSTERS WAS DILUTED WITH DECALCIFIED SERUM FROM NORMAL CONTROL ROOSTERS, AND THE PRODUCT SUBMITTED TO ULTRAFILTRATION. ($Ca^{++}{}_D$ REPRESENTS DIFFUSIBLE CALCIUM; CaProt = TOTAL Ca − $Ca^{++}{}_D$ = PROTEIN-BOUND CALCIUM)

	Estrogenized serum in mixture, per cent			
	100	75	50	25
Total protein, gm./100 cc.	7.4	6.55	5.7	4.85
Total calcium, mg./100 cc.	103.5	77.2	51.5	25.7
$Ca^{++}{}_D$, mg./100 cc.	7.2	4.5	3.6	2.3
CaProt, Mg Ca/100 cc.	96.3	72.7	47.9	23.4
Protein/CaProt	.077	.090	.119	.207
$1/Ca^{++}{}_D$	.139	.222	.278	.435

From these data and from those from a similar experiment, there is derived graphically (Fig. 4) the equation:

$$\frac{\text{Protein}}{\text{CaProt}} = \frac{1}{50} + \frac{0.4}{Ca_D{}^{++}}$$

Greenberg's constants: A = 50.0; B = 0.4
Maximum binding capacity of mixed serum proteins = 50 mg. Ca/gm. protein = 125 mM/100 grams.

the calcium-combining capacity of various proteins, by a procedure we have found to be inapplicable to the serum of estrogenized roosters, owing to the denaturation of phosphoprotein resulting from the addition of aqueous solutions containing NaCl and $CaCl_2$.

The calculations listed in Table 2 are based on dilution of the serum of estrogenized roosters with serum from normal roosters, following partial decalcification of the normal serum by adsorption on granular lead phosphate. We have found no indication that this treatment has any denaturing effect upon the phosphoproteins of the estrogenized animals. While this method does not permit any variation in total protein content, it does provide for varying the total calcium content. Under these conditions the diffusible calcium of the mixed serum remains approximately constant at about 6 mg./100 cc. In the absence of a suitable method for determination of Ca^{++}, total diffusible calcium (Greenberg's $Ca_D{}^{++}$) has been used as an approximation of Ca^{++}. Since not all of the variables are under strict control, and since in any case the calculations are made on a mixture of proteins, of which the X_1 component is relatively small in amount, but contributes a large share of the calcium-combining capacity, the results as presented are to be regarded only as an approximation of the ability of the phosphoproteins to form complexes with calcium.

Table 2 shows the results of an experiment in which pooled serum from estrogenized roosters was diluted with varying amounts of decalcified serum from control roosters. Tables 2 and 3 present calculations of the *capacity* factor of the proteins: serum globulin, serum albumin, casein, and the mixed proteins of the serum of the estrogenized rooster. Calculations on serum albumin and globulin in Table 3 are from Carr (9), casein figures are from McLean and Hastings (3), and those from the serum of the

TABLE 3. CALCIUM-COMBINING CHARACTERISTICS OF VARIOUS PROTEINS

	Capacity, mM Ca/100 gm.	Affinity, $pK_{Ca\,Prot}$
Serum globulin	4	$10^{-2.22}$
Serum albumin	6	$10^{-2.13}$
Casein	25	$10^{-2.50}$
Mixed proteins from serum of estrogenized rooster	125	$10^{-2.32}$

estrogenized rooster are from data obtained in the present study. Calculations of the *affinity* factor represent the values obtained for $pK_{Ca\,Prot}$ by the equations of McLean and Hastings, with calcium-combining capacity of the proteins represented in molar terms. From Table 3 it is seen that whereas purified serum globulin and serum albumin have a relatively low capacity for combination with calcium, this property is markedly increased in casein, the prototype of a phosphoprotein, and increased again, by a further factor of five, in the mixed proteins of the serum of the estrogenized rooster. On the other hand, the affinity of the combining groups of proteins for calcium, as measured by the dissociation constant, $pK_{Ca\,Prot}$, is not greatly different if at all in the proteins listed in Table 3. The significance of these observations will be discussed further below.

Colloidal Calcium Phosphate.—Analysis of the densely packed sedimented material found in the bottom of segment IV indicated that all or most of the excess calcium in the serum could be accounted for as bound to protein. If a small amount existed in any other form it could not be identified as colloidal inorganic calcium phosphate, in spite of the fact that inorganic phosphate levels increased as much as four times after injection of estrogen. In one experiment the proteinaceous syrup and sediment in the bottom of the partition cell was precipitated by adding an equal volume of trichloroacetic acid. The level of inorganic or protein-free phosphorus in the sediment was 26.0 mg.% and in the supernatant fluid after this treatment was 13.0 mg.%. It is assumed that no appreciable amount of colloidal calcium phosphate was present, for if it has been it would have been soluble in trichloroacetic acid and would have been represented by a much greater amount of phosphorus than the 13.0 mg.% found.

Paper Chromatography for Amino Acids.—Whole serum acid hydrolysates, and ultrafiltrates of control and estrogenized serum showed interesting patterns of the amino acids. The area and intensity of staining for serine was markedly increased following estrogen treatment. Galactosamin phosphate, a component of the serum of the untreated roosters, was generally absent in estrogenized serum. These observations suggest that serine is the characteristic amino acid and correspond to chemical analyses (10) (11) showing that phosphoserine is the predominant building stone of egg yolk and perhaps therefore of the X_1 component of the serum as well.

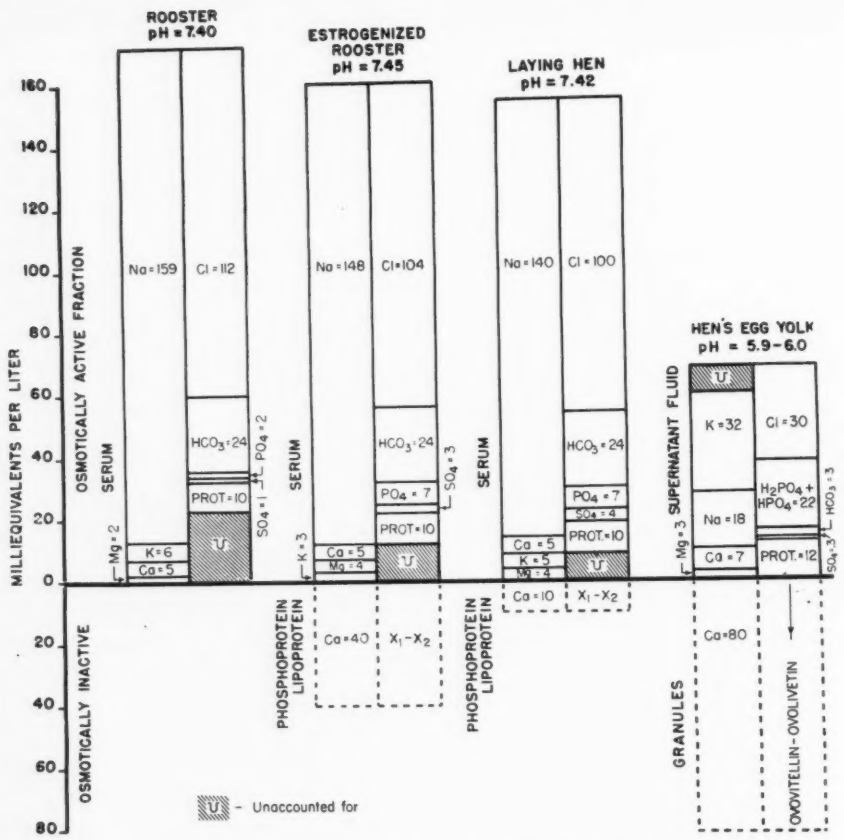


FIG. 5. Diagram showing the pH and electrolyte equilibria of the serum of normal and estrogenized roosters, and of the laying hen, compared with the supernatant fluid separated from egg yolk by ultracentrifugation. It may be assumed that the calcium bound by protein, as shown below the base line, was electrically neutralized and osmotically inactive, since no significant changes in electrolyte equilibria were produced by estrogenization.

The Egg Yolk.—Figure 5 summarizes the results of chemical analyses on blood and on the egg yolk. The yolks of 6 hens' eggs were separated into supernatant fluid and solids by centrifugation at 30,000 RPM for 4 hours. The supernatant fluid was analyzed for calcium and for the other principal cations and anions found in the fluids of the body. The fluid portion of the yolk contained 14 mg. to 24 mg.% calcium, a large part of which was ultrafilterable. The sodium content was greatly reduced, to a range of 15–20 meq. per liter as compared with 135 to 150 for plasma. Potassium increased in concentration in the egg yolk, as it does in other intracellular fluids. Chloride fell, but the concentration of inorganic phosphate rose from

9.0 meq. per liter in plasma to 38 meq. in egg yolk fluid. The bicarbonate content fell from 24.0 meq. per liter in plasma to less than 3.0 meq. in the fluid of the egg yolk. The result was that the pH fell from 7.45 in the hens' plasma to as low as 5.92 in the egg yolk.

The solid material of the egg yolk represented approximately 50% of the volume of the yolk (12) compared with approximately 10% of the volume of the serum of the estrogenized rooster. The concentration of protein in the whole yolk is approximately 14 gm. per 100 cc. On centrifugation, the granules that are separated from the fluid, after washing, contained water and approximately 25.5 gm. of total protein per 100 cc. After repeated wash with distilled water, filtering and centrifuging at 70,000 g. for two hours, the granules consisted of highly concentrated samples of phosphoprotein (X_1) and lipoprotein rich in sulfur (X_2). Of the 25.5 gm.% of total protein, 3.5 gm. was phosphoprotein (X_1) and the remainder, the sulphur-rich phospholipid lipoprotein (X_2). The same washed material contained 200 mg. calcium per 1.5 gm. of yolk granule protein (phosphoprotein and lipoprotein). For comparison, the blood of the laying hen contained 20 mg.% of calcium, bound to 0.3 gm. of X_1. It appears that the ovary removed the phosphoprotein from the blood together with a large part, if not all, of its complement of calcium. Ovalbumin and the other lipoproteins were found in the liquid compartment of egg yolk but these require further investigation.

DISCUSSION

The ultracentrifugal analyses herein reported afford clear evidence that the dramatic increase in the serum calcium level of the estrogenized rooster is associated chiefly with phosphoproteins not present in the blood of the untreated rooster, but found in the serum of the laying hen and appearing in considerable quantities in the serum of roosters treated with large doses of estrogenic hormone. Although the rooster was chosen for these studies, our interest is primarily in the physiology of oviparous animals; the rooster serves as a suitable test animal for the reason that the effects of exogenous estrogen can be controlled without the interference contributed by the endogenous estrogen of the laying hen.

Our data show that the phosphoproteins under study have a very large capacity for combination with calcium. For technical reasons it has not been possible as yet to arrive at an accurate estimate of either the capacity or the affinity of these proteins, in a pure state, for this cation. It appears, however, that the affinity of these proteins for calcium, as measured by the dissociation constant, pK_{CaProt}, does not differ materially from that of other proteins previously studied, including serum albumin, serum globulin, and casein; there are simply more calcium-combining groups per molecule or per unit weight of the phosphoproteins than there are in the usual serum proteins. It is suggested herein, but not proved, that the high

capacity of the protein molecule for calcium-binding is contributed by the phosphate groups of the phosphorylated amino acid, phosphoserine. It is also assumed that the carboxyl groups of the proteins, responsible for the binding of calcium by proteins which lack phosphate groups, also participate in the combination of the phosphoproteins with calcium.

There has been an implicit assumption in the literature that the binding of calcium by the phosphoproteins of the serum of laying birds is associated physiologically with the transport of the calcium required for the egg shell. It now appears, however, that the correlation is with the egg yolk rather than with the egg shell, and that it is more meaningful to regard the hypercalcemia as serving for the transport of the serum proteins. This view is supported by our observation, as yet unpublished, that both the hypercalcemia and the production of serum phosphoproteins are readily induced in the frog and in fish by administration of estrogen; in both cases there is the requirement for egg yolk production, but none at all for the deposition of an egg shell. Moreover, in spite of numerous trials, no one has succeeded in inducing a significant hypercalcemia or the appearance of a serum phosphoprotein in mammals, comparable to that observed in birds, frogs or fish, by the administration of estrogen, even though in mice the production of medullary bone under the influence of estrogen resembles that in birds. Here the correlation is: no egg yolk production—no serum phosphoprotein—no hypercalcemia. We believe, therefore, that we are correct in attributing the hypercalcemia of birds and oviparous cold-blooded animals to the requirement of the circulating phosphoproteins for calcium. It is of interest that the male of each oviparous animal studied retains the ability to exhibit hypercalcemia and the production of serum phosphoproteins, even though the formation of the end product of the reaction—the egg yolk—is an impossibility. Female amphibians and fish show hypercalcemia and serum phosphoproteins in the preovulatory phase of the reproductive cycle, but do not, of course, produce calcified egg shells.

Endocrine Interrelationships.—The literature on the subject of hypercalcemia in the laying hen or estrogenized bird contains many different but interdependent observations. The first correlation by early investigators was with the large increase in phosphorus and as a result colloidal calcium phosphate was postulated as one of the components of the plasma (13–16); this idea is still receiving support (17). The next observation was the increase in the level of the total phosphorus and the appearance *de novo* of phosphoprotein in the blood (18–22). The next concerned the reproductive cycle and the causal effects of both endogenous and exogenous estrogen (23–36). The next described the hyperproteinemia and specific electrophoretic components formed in the plasma in response to estrogen treatment (37–46). More recently, the hypercalcemia has been correlated with the transport of a phosphoprotein-lipoprotein complex, X_1–X_2, formed

by the liver and supplied to the ovarian follicles for construction of the yolk proteins (1). In the course of the normal cyclical changes in the composition of the serum of the laying hen the level of the total calcium may rise to a peak of 30.0 mg.% or higher. It is now quite clear that this is related to the production of estrogen by the graaffian follicles. Riddle (33) estimated that the ovaries of the domestic hen produce estrogenic activity equivalent to 3.0 mg. of estradiol benzoate per day.

Extirpation and replacement therapy experiments have failed to show any correlation between the cyclical hypercalcemia of birds and the function of other glands, such as the pituitary or adrenals. Parathyroidectomy reduces the level of calcium ion in the blood and results in the formation of eggs with uncalcified shells (47).

The Egg Shell.—We have attributed the hypercalcemia of birds, during the egg-laying cycle, to the requirement of the circulating phosphoproteins for calcium, rather than to the need for transport of this mineral for the formation of the egg shell. As one of the reasons for this view we have cited the fact that fish and amphibians exhibit the same hypercalcemia and phosphoproteinemia without forming calcified egg shells. This does not, however, exclude the frequently entertained but quite unproven possibility that the hypercalcemia in the bird contributes to the transport of calcium for the egg shell (48, 49). Our present view is that all or most of the protein-bound calcium is drained from the blood by the ovarian follicles in the formation of the egg yolk, and that egg shell formation and the calcification of medullary bone is sustained by the calcium ion content of the plasma. In the bird, however, as in the mammal, the calcium ion content of the plasma is buffered by the presence of readily available protein-bound calcium, which may make some contribution to the egg shell. That the calcium ion concentration is of first importance, however, is indicated by the fact, above referred to, that the reduction in calcium ion concentration in the blood in birds following parathyroidectomy results in the formation of eggs with uncalcified shells, without affecting the amount of protein-bound calcium in the plasma (50). The X_1–X_2 precursors of yolk protein hold the highest priority on the supply of calcium. Low calcium diets, fortified with vitamin D, produce in succession: thin-shelled eggs, shell-less eggs, small eggs, and then no eggs. In mice and in cortisone-treated birds the female sex hormone can produce endosteal bone in the absence of both hypercalcemia and phosphoproteinemia; the calcification of medullary bone is thus not dependent upon an excess of protein-bound calcium.

Calcium and Protein in the Egg Yolk.—The egg yolk may be regarded as a selected concentrate of the serum of the laying hen. The transfer of protein across the capillary and vitellin membranes and into the yolk sac is a complicated process, involving the action of special enzymes such as phosphoprotein phosphatase. Also observed are pinocytosis and extreme changes in pH, in capillary permeability, and in electrolyte equilibria. The

composition of the yolk fluid, as compared with the plasma of the hen, has been noted above, and is summarized in Fig. 5. Associated with these changes the soluble complexes of X_1 and X_2 were transformed into aggregates or insoluble granules. The relationship between these granules and the time honored embryologic terms ovovitellin and ovolivetin require review and definition by special physical and chemical analyses. For this purpose, the process of aggregation of the yolk granules, together with the enzymatic mechanisms and processes concerned with deposition of lipid and carbohydrate substances are under investigation at the present time, and will form the subjects of further communications.

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THE ENHANCEMENT OF RELAXIN-INDUCED GROWTH OF THE PUBIC LIGAMENT IN MICE[1]

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ABSTRACT

Using the x-ray measurement of interpubic distance as an assay procedure, it was found that purified relaxin which is of low potency in mice can be enhanced in activity by delaying its absorption from the site of injection. Of the depot media and binding agents which were tested, the most effective was a suspension of lyophilized relaxin in 5% beeswax and oil, which produced a 65-fold increase in assay potency. In contrast, the mouse-active fraction of sow ovaries, which is notably less soluble than relaxin, was not potentiated by the depot medium. It was therefore concluded that the growth response of the pubic ligament can be obtained with depot relaxin, and that the mouse-active fraction represents a more slowly mobilized form of relaxin.

The relationship of these findings to the interpretation of bioassay data was discussed, and a modified procedure was suggested to provide a quantitative assay of relaxin in mice.

DURING the course of studies in relaxin bioassay, it was noted that purified relaxin was surprisingly ineffective in the production of pelvic ligament growth in mice, although crude relaxin extracts had been quite potent (1, 2). A more powerful stimulus for ligament growth was later detected in a protein-like, relatively insoluble, ovarian fraction which was easily separable from relaxin but contained trace amounts of relaxin (3). In addition, chemical and pharmacological studies suggested that the two ovarian fractions were related, and that the mouse-active fraction may represent protein-bound relaxin. This paper presents evidence in support of this hypothesis, and proposes a modification of the roentgenographic bioassay method in mice to allow a quantitative assay of purified preparations of relaxin.

MATERIALS AND METHODS

Bioassay procedure: Mature, virgin, female mice were obtained from Rockland Farms, New York, and were used for one assay when they weighed between 22 to 26

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gm. each. Following seven to eight days pretreatment with 1 μg. of estradiol-17β daily, injected subcutaneously in sesame oil, the animals were anesthetized with 2.5 mg. of sodium pentobarbital injected intraperitoneally in 0.2 ml. of 1% saline. Code numbers were assigned and x-ray photographs of the pelvis were obtained by the method of Kliman, Salhanick, and Zarrow (2). A single dose of the test substance was then injected subcutaneously and roentgenograms were again taken 24 hours later. The increment in the separation of the pelvic bones at the pubic symphysis was then measured by projection of the x-ray films onto millimeter ruled graph paper at a constant magnification of ten diameters. Multiple dose levels of unknown and standard preparations were tested at the same time, and the data were subjected to conventional statistical analysis (4, 5). The results were expressed in terms of the ratio of potency, *M*, of the unknown compared to the standard preparation. The limits of error for the potency ratio were rigorously calculated for a probability of 95 chances out of 100, that the true ratio was enclosed by those limits.

Ovarian extracts: All preparations were derived from the ovaries of pregnant sows, and the relaxin contents were determined by the palpation assay in guinea pigs according to the Frieden and Hisaw modification (6) of the Abramowitz *et al.* method (7). Extracts R-12, G-111, and R-175 (Releasin) were obtained through the courtesy of Dr. Robert L. Kroc of Warner-Chilcott Labs. The mouse-active fraction, X-4, was isolated by two precipitations with 50% ethanol from a 5% saline extract of sow ovaries. The relaxin assays were performed in guinea pigs by Dr. Kroc, and the results were expressed in Guinea Pig Units (GPU) per mg. of salt-free solids.

Injection media: Unless stated otherwise, all injections were given subcutaneously in 0.1 ml. of medium. The test doses of the relaxin standards were dissolved in 0.9% saline. The mouse-active fraction was suspended in 0.9% saline and was mixed prior to injection.

The gelatin depot medium was composed of 20% gelatin and 30% propylene glycol in water. Relaxin was dissolved in the medium and the solution was warmed to 60° C before use.

Relaxin tungstate was prepared fresh by the addition of 4% sodium tungstate (1 volume) to a relaxin solution (1 volume), and acidification to a final pH of 4.5 by the addition of 2 volumes of KCl (0.05N)-HCl (0.01N) buffer. A white, flocculent precipitate appeared, and was suspended in the supernatent solution prior to injection.

Polyvinylpyrrolidone was purchased from Delta Chemical Works, Inc., New York. The powder was dissolved in 0.9% saline at concentrations of 5, 10 or 20% by weight. Relaxin was then dissolved in this medium and was injected at room temperature.

The preparation of beeswax-oil medium was based upon the methods of Code and Varco (8), Romansky and Rittman (9), and Bruce and Parkes (10). Pure beeswax was flaked and then added to sesame oil to make 5% wax by weight. The mixture was heated at 65° C for 48 hours to dissolve the wax. The medium can be cooled since it will liquify within 15 minutes upon reheating. The extracts to be assayed were first lyophilized, and the resulting powders were dispersed in the warm medium with the aid of a glass tissue-grinder. The mixtures were reheated to 60° C and stirred before injection.

RESULTS

Gelatin injection medium:

Although a previous attempt to alter relaxin activity with 10% gelatin had been unsuccessful (3), it seemed worthwhile to test a more slowly absorbable mixture containing 20% gelatin and 30% propylene glycol, and to use multiple dose levels for better accuracy. Preparation G-111,

TABLE 1. EFFECT OF A GELATIN INJECTION MEDIUM ON RELAXIN ASSAY IN MICE

No. of mice	Dose assayed		Response in mm. ±S.E.	Statistics*
	Mg.	GPU		
	A. Relaxin preparation G-111, diluted with saline			
8	0.11	12.5	0.24 ±0.09	$b = 0.762$
7	0.23	25	0.43 ±0.15	$s = 0.031$
7	0.45	50	0.70 ±0.18	$\lambda = 0.041$
	B. G-111, diluted with 20% gelatin			
8	0.11	12.5	0.41 ±0.12	$b = 0.615$
7	0.23	25	0.67 ±0.14	$s = 0.060$
8	0.45	50	0.78 ±0.14	$\lambda = 0.098$
7	0.9	100	1.21 ±0.27	

Potency Ratio, M, for $\frac{\text{Gelatin}}{\text{Saline}} = 1.72\ (1.37 - 2.14)$ at $P = 0.95$.

The difference between slopes (b) was not significant: $P = 0.15$.

* Equation of line, $y = a + bx$.

b = slope of the line assigned by method of least squares.

s = standard error of estimate; derived from

$$\sqrt{\frac{\Sigma(y - Y \text{ calc.})^2}{n' - 2}}$$

where Y calc. is the value of Y calculated from the equation of the line, and n' is the number of doses.

λ is the error of x as estimated from y and is equal to s/b.

with a potency of 111 guinea pig units of relaxin per mg. dry weight, was dissolved in the gelatin medium at a concentration of 1000 GPU per ml., and was then heat-sterilized. In order to eliminate any harmful effects of this procedure, both the saline and the gelatin diluted samples were made from the gelatin stock solution. The 50, 25, and 12.5 GPU doses of the saline standard therefore contained 10, 5, and 2.5% of gelatin, respectively. Despite this handicap, a significant increase in biological activity was demonstrated with the use of the gelatin injection medium (Table 1). The responses in a total of 52 mice tested at three dose levels of the standard (saline dilutions) revealed a potency ratio for gelatin/saline of 1.72 with a range of 1.37 to 2.14 at a P value of 95%. The statistics for the dose-response curves showed comfortably low values for the coefficient of variability (λ) and no significant difference between slopes (b), indicating that the assay comparison was valid.

Relaxin tungstate:

A purified relaxin preparation, R-175, with a specific activity of 175 guinea pig units per mg., was dissolved in saline at a concentration of 20 mg. (3500 GPU) per ml. Dilutions of the stock solution were then made with either saline or sodium tungstate solution. All solutions were acidified to pH 4.5 before injection. The tungstic acid treated relaxin formed a white, flocculent precipitate which was easily suspended by shaking. The

TABLE 2. EFFECT OF A TUNGSTIC ACID INJECTION MEDIUM ON RELAXIN ASSAY IN MICE

No. of mice	Dose assayed		Response in mm. ±S.E.	Statistics*
	Mg.	GPU		
	A. Relaxin preparation R-175, in saline			
7	0.25	44	0.19 ±0.06	$b = 0.852$
7	0.5	88	0.37 ±0.08	$s = 0.062$
8	1.0	175	0.70 ±0.13	$\lambda = 0.072$
	B. R-175 in 1% tungstic acid			
7	0.25	44	0.41 ±0.18	$b = 0.929$
7	0.5	88	0.77 ±0.22	$s = 0.065$
7	1.0	175	0.97 ±0.16	$\lambda = 0.070$

* Defined in Table 1.

Potency Ratio, M, for $\frac{\text{Tungstate}}{\text{Saline}} = 2.02\ (1.57 - 2.60)$ at $P = 0.95$.

The difference between slopes is not significant: $P = 0.40$.

results of a comparison assay of the two preparations in a total of 43 mice are recorded in Table 2. A twofold increase in relaxin activity was achieved with the tungstate medium, and statistical analysis verified that the bioassay values were reliable.

Polyvinylpyrrolidone medium:

Significant but still minor changes in relaxin activity had been observed, and it was hoped that the binding properties of polyvinylpyrrolidone (PVP) would prove useful (Schubert, 1948). Two consecutive, submaximal doses of relaxin R-175 were assayed with saline, or 5, 10, and 20% PVP solution. The 24-hour response to each dose was obtained (Table 3). It is

TABLE 3. THE OPTIMAL CONCENTRATION OF POLYVINYLPYRROLIDONE FOR A RELAXIN INJECTION MEDIUM

No. of mice	Dose assayed, R-175			Response in mm. ±S.E.	
	Mg.	GPU	Medium	One dose	Two doses
8	0.2	35	Saline	0.20 ±0.10	0.25 ±0.09
8	0.2	35	5% PVP	0.30 ±0.10	0.43 ±0.13
7	0.2	35	10% PVP	0.54 ±0.23	1.00 ±0.32
8	0.2	35	20% PVP	0.60 ±0.13	1.05 ±0.11

evident that a significant potentiation of relaxin was afforded by the use of PVP, and that a concentration of 10% PVP appears to be adequate to produce this effect.

The degree of enhancement with 10% PVP was examined in two separate assays, the results of which are summarized in Table 4. The dose-response data were satisfactory and the increase in relaxin potency was found to be more than sixfold.

TABLE 4. EFFECT OF 10% POLYVINYLPYRROLIDONE MEDIUM ON RELAXIN ASSAY IN MICE

No. of mice	Dose assayed		Response in mm. ±S.E.	Statistics*
	Mg.	GPU		
		A. Preparation R-175 in saline		
7	0.4	70	0.33 ±0.10	$b = 0.971$
7	0.5	88	0.29 ±0.09	$s = 0.099$
6	1.0	175	0.68 ±0.25	$\lambda = 0.102$
		B. R-175 in 10% PVP		
6	0.1	17.5	0.55 ±0.21	$b = 1.06$
7	0.2	35	0.83 ±0.23	$s = 0.094$
8	0.1	17.5	0.48 ±0.11	$\lambda = 0.090$
8	0.2	35	0.69 ±0.18	

* Defined in Table 1.

Potency Ratio, M, for $\frac{\text{PVP}}{\text{Saline}} = 6.28\ (4.50 - 8.73)$ at $P = 0.95$.

There is no significant difference between slopes ($P = 0.60$).

Beeswax in oil medium:

At this stage, it was still not possible to account for the differences in assay values for the two ovarian fractions by pharmacological factors alone. The experience of Bruce and Parkes with beeswax-oil enhancement of corticotropic activity (10) encouraged us to continue studies with depot relaxin, using a similar injection medium.

A partially purified, low-potent, relaxin preparation (R-12) with a specific activity of 12 GPU per mg. was lyophilized and suspended in 5% beeswax and oil. An aqueous solution of the same extract was assayed at the same time. The results appear in Table 5. In terms of response, converted to mouse units by the assay method of Kliman, Salhanick, and Zarrow (2), the wax-oil medium elicited a 12.5-fold increase in relaxin activity.

Since partially purified relaxin retains a moderate degree of activity in

TABLE 5. EFFECT OF WAX-OIL INJECTION MEDIUM ON THE ASSAY OF LOW-POTENT RELAXIN IN MICE

No. of mice	Dose assayed, R-12			Response in mm. ±S.E.	Mouse units per mg.
	Mg.	GPU	Medium		
5	1	12	Saline	0.32 ±0.19	
5	2	24	Saline	0.54 ±0.20	0.4
6	4	48	Saline	0.96 ±0.39	
8	0.5	6	Wax-oil	1.00 ±0.11	5.0
9	1.0	12	Wax-oil	1.36 ±0.24	

Based on unit of response, $\frac{\text{R-12 in wax-oil}}{\text{R-12 in saline}} = 12.5$.

TABLE 6. EFFECT OF WAX-OIL INJECTION MEDIUM ON THE ASSAY OF PURIFIED RELAXIN IN MICE

No. of mice	Dose assayed, R-175			Response in mm. ±S.E.	Statistics*
	Mg.	GPU	Medium		
10	0.25	44	Saline	0.51 ±0.09	$b = 0.79$
10	0.5	88	Saline	0.78 ±0.20	$s = 0.025$
10	1 0	175	Saline	0.99 ±0.16	$\lambda = 0.031$
8	0.00156	0.27	Wax-oil	0.08 ±0.03	$b = 1.045$
8	0.00313	0.55	Wax-oil	0.43 ±0.18	$s = 0.025$
9	0.00625	1.09	Wax-oil	0.71 ±0.23	$\lambda = 0.024$

* Defined in Table 1.

Potency Ratio, M, for $\frac{\text{R-175 in Wax-Oil}}{\text{R-175 in Saline}} = 65.6\ (59.4 - 72.4)$ at $P = 0.95$.

The difference between slopes is not significant ($P = 0.40$).

the mouse bioassay, it seemed desirable to study a more purified relaxin preparation than R-12. A sample of relaxin R-175 was lyophilized and then assayed in saline and in beeswax-oil. Pelvic ligament growth was measured in a total of 55 mice. A quite formidable potentiation of relaxin, approximately 65-fold, was recorded (Table 6). A dose of only 6.25 μg. in wax-oil proved to be as effective as 500 μg. in saline solution. The depot preparation produced one mouse unit of response with the injection of one guinea pig unit of relaxin, thereby exceeding the effectiveness of crude relaxin extracts which contain only 0.1 mouse unit for each guinea pig unit of relaxin.

To rule out the possibility that the mouse-active fraction was present in all preparations and was potentiated by some means such as protection from tissue inactivation, a concentrate of mouse-active fraction was studied. Extract X-4 was assayed at effective dose levels in saline suspension, and at slightly lower dose levels in wax-oil in an attempt to detect even a slight enhancement (Table 7). To the contrary, an actual decrease in assay response occurred. It is also evident that the medium itself was not biologically active.

A summary of the effects of the depot media tested appears in Figure 1.

TABLE 7. EFFECT OF WAX-OIL INJECTION MEDIUM ON MOUSE-ACTIVE-FRACTION ASSAY IN MICE

No. of mice	Dose assayed, X-4		Response in mm. ±S.E.	Mouse units per mg.
	Mg.	Medium		
6	5	Saline	0.48 ±0.17	0.20
7	10	Saline	0.99 ±0.19	
7	1	Wax-oil	0.00 ±0.04	<0.10
7	2	Wax-oil	0.11 ±0.12	

Based on unit of response, $\frac{\text{X-4 in wax-oil}}{\text{X-4 in saline}} = <0.5$.

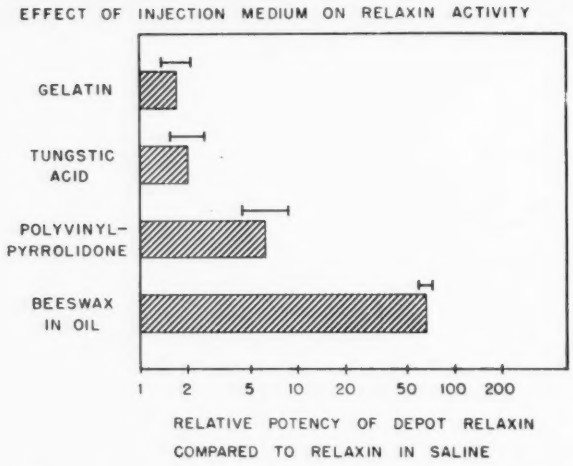

FIG. 1. The enhancement of relaxin action in mice by a variety of depot injection media. The aqueous standard solution is arbitrarily assigned a value of one. The brackets enclose the 95 per cent limits for the potency ratio of unknown/standard.

DISCUSSION

When purified relaxin proved to be a weak stimulus to pubic ligament growth in mice, the effect of the purification process on the activity of crude ovarian extracts was investigated. At first, with the isolation of an ovarian fraction which was potent for mice but not for guinea pigs, the existence of a species specific "mouse relaxin" was postulated (1). This explanation was soon modified in the attempt to account for a number of discrepancies. It seemed unlikely that the blood serum of pregnant rabbits and the ovaries of pregnant sows both contained hormones which were species specific for mice and guinea pigs. Also, the ratio of relaxin content to mouse relaxin content was curiously similar for extracts from these two sources, and intermediate dose levels of relaxin produced a typical dose-response curves in the mouse bioassay (3), although the higher dose levels which were tested in this study gave reliable assay results. The demonstration that intraperitoneal injection could reduce the assay activity of relaxin or of mouse-active fraction in mice confirmed the importance of pharmacological factors, but accounted for not more than a twofold alteration in activity (3).

The mouse-active fraction of sow ovaries is notably less soluble than relaxin, and is not in a well concentrated state as yet so that it must be injected in aqueous suspension. Relaxin, on the other hand, is highly soluble (6), and is rapidly absorbed from the tissues and rapidly eliminated from the blood (11). Accordingly, various injection media were tested with the intention of delaying the absorption of relaxin from the injection site. The results bear a close resemblance to those found during the development of

long-acting ACTH preparations (12). A water soluble medium, 20% gelatin, and a relaxin tungstate complex each increased the effectiveness of relaxin by about twofold. The familiar plasma substitute, polyvinylpyrrolidone (PVP), has an unusual ability to bind proteins and certain dyes (13). The use of PVP in concentrations of 10 or more per cent in water led to a sixfold enhancement of purified relaxin. This medium is the easiest of all to prepare and inject since it is aqueous and flows well at room temperature.

Mixtures of beeswax in oil have been used to prolong the action of histamine (8), desoxycorticosterone acetate (14), penicillin (9), curare (15), ACTH (10) and other drugs and hormones. A suspension of lyophilized relaxin in 5% beeswax in oil proved to be highly effective for the induction of pelvic ligament growth. The degree of enhancement for two relaxin preparations of different potency appeared to be correlated with the relaxin content so that one to two guinea pig units elicited a response equivalent to one mouse unit. Since preparation R-175 was enhanced 65-fold while the mouse-active fraction lost 50% or more of its activity in this medium, the overall alteration in relaxin assay amounted to at least 130-fold. It is also significant that this depot medium caused purified relaxin to surpass the effectiveness of crude relaxin-containing extracts of sow ovary. Previous comparisons of a group of ovarian extracts have shown a 600-fold discrepancy, on the basis of guinea pig units, between mouse-active fraction and relaxin when tested in the mouse (3). Although one extract, 52-2, appeared to show an even greater change, it was not available in sufficient quantity to assay thoroughly and no other preparations have exceeded the 600-fold limits. There thus remains a 4- to 5-fold difference in assay effectiveness that cannot be directly accounted for, although the errors inherent in the comparison of two different bioassay procedures are probably large. It is also possible that the wax-oil medium releases relaxin too slowly for optimal effects, which would also explain the decreased effectiveness of mouse-active fraction in the depot medium. The actual requirements of pubic ligament growth for relaxin from hour to hour are unknown, and the 8-hour lag period which follows the injection of either ovarian fraction into mice (2) may well represent a period during which high blood levels of the hormone are not utilized. In any event, it is clear that the pharmacological properties of the extracts to be assayed are of paramount importance. It also appears that the two ovarian fractions are closely related and that the mouse-active fraction represents a slowly mobilized form of relaxin.

What is the nature of the mouse-active fraction? This substance contains small amounts of relaxin, gives the characteristic tests for protein, and retains biological activity when it is solubilized. Mouse-active fraction could be a high molecular weight analogue of relaxin or a simple complex of relaxin with tissue proteins. On the basis of chemical studies, Frieden

and Hisaw (6) concluded that relaxin is present in a complexed form within the ovary. We have recently found that purified relaxin binds poorly to albumin or to protamine and zinc, which suggests that the mouse-active fraction is more than a simple binding product of relaxin. The physiological role of the mouse-active fraction is uncertain as yet, but it may be that it is a secretion form of the relaxative hormone and that the purification process splits off the biologically active sub-unit, relaxin.

Several procedures have been proposed for the bioassay of relaxin. The guinea pig palpation assay (6, 7) has proved reliable in the past, but is inherently subjective in character and yields only qualitative estimates of activity. Studies of electrometric dilution potential changes in the pubic symphysis of the guinea pig are promising, although the data published thus far reveal a response curve which is unfavorable for assay since fourfold gradations in dose levels were used (16). The first attempt to develop a roentgenographic assay for relaxin in guinea pigs yielded results which were too variable for any practical assay (17). Marois, Nataf, and Marois (18) used higher doses of relaxin and a 12-hour response period, and obtained separations of the pubic symphysis of guinea pigs, as measured by x-ray, which may prove useful for assay, but a typical dose-response curve has not been reported. X-ray studies of pubic separation in mice revealed that purified relaxin was relatively ineffective compared to crude extracts (1), and this method of assay was reserved for the study of mouse-active fractions of relaxin extracts (2). Dorfman *et al.* (19) proposed an assay in mice, based on the low level of response to multiple doses of relaxin, but reported a dose-response curve with a high coefficient of variability (λ) of 40%, which is unfavorable for assay.

Now that the activity of relaxin can be routinely enhanced in mice, the method of assay proposed by Kliman, Salhanick, and Zarrow (2) modified to include the use of depot media appears to be the one of choice.

Acknowledgments

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BLOCKADE OF OVULATION IN THE RAT BY MESENCEPHALIC LESIONS[1,2]

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ABSTRACT

To determine whether or not the midbrain is involved in the control of ovulation, large mesencephalic lesions have been placed in 76 rats on the morning of proestrus, prior to the "critical period." When examined approximately 24 hours later, 52 of the lesioned rats had failed to ovulate as evidenced by the lack of tubal ova. Thirty-seven of these "blocked" animals were maintained until the morning of the second day after lesioning and 22 were still blocked; the remaining 15, however, had tubal ova, indicating that ovulation had merely been delayed 24 hours. Fourteen rats which failed to ovulate by the second day following lesioning were examined at autopsy on the third day; 13 of these still had not ovulated. A comparison of the lesions responsible for the three types of results, i.e. no blockade, transient blockade or blockade for two or three days, revealed that the lesions associated with the failure to ovulate for two or three days involved an area of the brain stem that was not destroyed by the other lesions. The area that appears to be essential to ovulation is located ventro-medially in the mesodiencephalic junction. The portion of the mammillary peduncle located rostrally to the interpeduncular nucleus seems to fulfill most completely the anatomical criteria imposed by the lesion data. This and adjacent structures are discussed as to a possible role subserved in ovulatory mechanisms. No specific behavioral deficit was found to signal an "effective" lesion. The finding that massive destruction of the midbrain tegmentum and the resulting somnolence are compatible with ovulation seems to rule out the possibility that neural mechanisms underlying behavioral arousal are crucially involved in this neuroendocrine phenomenon. It may be that some other midbrain-diencephalic "activating system," unrelated to sleep-wakefulness, controls the cyclic release of pituitary ovulating hormone in this "spontaneously" ovulating form.

MANY pharmacological agents have been demonstrated to block ovulation in the rat if administered so as to be effective during a two-hour "critical period" on the day of proestrus (1). The mechanisms by which these various agents exert their effects are obscure. Sawyer *et al.* (2)

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have pointed out that ovulation-blocking doses of atropine, pentobarbital (Nembutal) and morphine have certain effects in common: they all produce high-amplitude slow waves in the electroencephalogram (EEG) and all raise the threshold of EEG arousal induced by stimulation of the midbrain reticular formation. On this basis, it was suggested that the midbrain reticular substance, as it is conceived to function in a diffuse arousal system (3), might be involved in the neurogenic phases of the ovulatory process. More recently, chlorpromazine (4) and ethyl alcohol (5), in doses which block ovulation, have been found to produce similar changes, and it has been suggested that these agents may also exert their ovulation-blocking effects through the reticular formation.

Further evidence implicating the midbrain in reproductive processes has been recently obtained by Kawakami and Sawyer (1, 6). Using castrate, estrogen-primed female rabbits with electrodes chronically placed in the brain, these workers have observed altered thresholds in the arousal system that parallel the sequential facilitatory and inhibitory actions of progesterone on the release of ovulating hormone.

The purpose of the present experiments has been to investigate more directly the possibility of midbrain involvement in gonadotrophic functions by studying the effects of mesencephalic lesions on ovulation in the rat. This form is well-suited for such studies because of the apparent encephalization of the neural elements essential to ovulation. Unlike the condition existing in reflex ovulators, there is no dependence upon the rapid triggering effects of genital stimulation that must be transmitted via afferent pathways traversing the midbrain. Therefore, effective mesencephalic lesions in the rat should be more specific in implicating this region in central mechanisms involved in the release of ovulating hormone.

In planning an approach with which to explore the effects of midbrain lesions on ovulation, it was anticipated that large lesions in this area of the brain stem would result in comatose animals. To avoid the complications to pituitary physiology that would be introduced by such a chronic condition, these experiments were designed to examine the short-term effects of mesencephalic lesions.

MATERIALS AND METHODS

Seventy-six mature Sprague-Dawley female rats, weighing between 230 and 280 gm., were used in these experiments. Controlled lighting, with 14 hrs. of artificial light per day, was used to establish a predictable two-hour "critical period" during which time the nervous system participates in the release of ovulating hormone (7). All animals were maintained under these conditions for at least three weeks before being used. The stages of the estrous cycle were determined by vaginal smears taken daily except Sunday between 8:30 and 9:00 A.M.

A Birtcher Blendtone electro-surgical unit was used as the source of a 2.7 meps. coagulating current which was applied for 6–10 seconds through two stainless steel insect pin electrodes. The electrodes were spaced 1 mm. apart and the tips were bared of insulation for a distance of approximately 1 mm.

Animals were selected on the morning of proestrus, anesthetized with ether, fixed in a stereotaxic instrument, and lesions produced as described above. All lesions were completed at least 90 minutes prior to the established "critical period." To obtain the necessary destruction in the transverse plane, three entries were made routinely into the brain with the pair of lesioning electrodes; one across the midline and one immediately on either side. Lesions were occasionally placed at several dorso-ventral levels, depending on the area of destruction desired.

Thirty-nine rats were killed with ether on the morning following lesioning and examined for tubal ova according to the methods described by Everett (8). Ovulation was considered to have been blocked if tubal ova were not found. Some ovaries were placed in SUSA fixative, imbedded in paraffin, serially sectioned at 10μ, stained with hematoxylin and eosin and examined histologically. In no case were the findings of such an examination contradictory to those obtained at autopsy.

In order to study more prolonged effects of midbrain destruction on the ovulatory process, 37 rats were maintained beyond the first day post-lesioning; 23 of these animals were autopsied after two days and 14 were autopsied after three days. The latter group allowed observation throughout the full three-day follicular cycle defined by Everett and Sawyer (9). This extension of the period of observation required a correlate of lesion-induced blockade of ovulation which did not necessitate bilateral ovariectomy at the first examination. Such a correlate was furnished by the observation of distended, fluid-filled, proestrous uteri in 14 of 15 rats that failed to ovulate in the series of animals autopsied 24 hrs. after lesioning. Similar uteri have never been seen in 150 animals examined following ovulation. Thus, distended uteri on the morning following proestrus and under the conditions imposed by midbrain lesions appear indicative of blockade of ovulation.

Twenty-four hours after destroying a portion of the midbrain, the rats were anesthetized with ether, the abdomen opened and a uterine horn examined. If, in a given animal, the uterus was distended, ovulation was considered to have been blocked and the incision was closed. If the uterus was not distended, an ovary was removed and examined for tubal ova. Most ovaries from this series of animals were processed as described previously and studied histologically to verify the findings obtained during the examination for tubal ova. Examination of one ovary is a valid assay for ovulation: only three out of 90 rats have been observed to ovulate unilaterally. If no ova were found in the tube, it was concluded that ovulation had failed to occur and the animal was saved for study on the following day. If ova were found, the rat was sacrificed. All animals studied beyond the first day after lesioning were maintained with periodic feedings of 5% dextrose in normal saline and milk, administered by stomach tube.

On the morning of the second day following lesioning the abdominal incisions were reopened under ether anesthesia and an ovary removed for microscopic examination. In the case of rats which had revealed distended uteri on the previous day, this step in the procedure resulted in unilateral ovariectomy. For those from which an ovary had been removed during the previous examination, this step amounted to total castration and they were sacrificed and the brains removed. Rats which survived the second day with a unilateral ovariectomy and a maintained block were retained until the succeeding day at which time they were sacrificed and the brains and remaining ovaries removed. This protocol is illustrated diagrammatically in Figure 1.

Because of the possibility that a behavioral deficit might be correlated with blockade of ovulation in animals with large lesions in the midbrain tegmentum, post-lesion behavior was observed with special attention devoted to the degree of spontaneous activity exhibited and the responses evoked by a variety of sensory stimuli. Righting reflexes and responses to nociceptive stimuli were tested routinely. Because several rats died with temperatures in excess of 41° C, rectal temperatures were taken at intervals

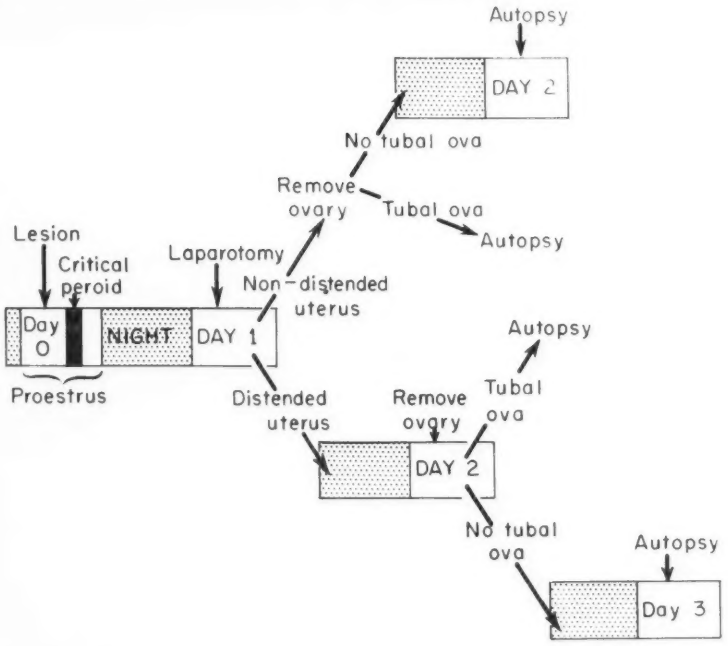

FIG. 1. Protocol followed to extend the period of observation of the effects of midbrain lesions on ovulation.

so that animals could be cooled by water-immersion if necessary. These emergency measures were carried out when temperatures exceeded 39° C.

A potential complication in the study of the effects of midbrain lesions on the release of ovulating hormone in the rat is related to the position of the pituitary. The hypophysis is large and underlies the midbrain and rostral pons. Therefore, in studying the effects of mesencephalic lesions, the possibility of a blockade of ovulation due to inadvertent pituitary damage must be considered and, if possible, eliminated. The following procedures were adopted in an attempt to control this problem.

1) *Examination of pituitaries at autopsy.*—Following the removal of brains, pituitaries were examined under a dissecting microscope. Extension of the lesion to the gland was easily recognized and when minimal it appeared as a slightly hemorrhagic flare. More extensive involvement was marked by discolored and coagulated tissue and, occasionally, electrode tracks. Animals with damaged pituitaries were discarded.

2) *Pituitary lesions.*—To check the possibility that unrecognizable pituitary damage resulting from edema and mechanical pressures might be responsible for the failure to ovulate, electrodes were deliberately inserted into the hypophyses of 10 rats and lesions produced. The location and extent of such lesions and the effects on ovulation were determined at autopsy on the following morning. Some glands were sectioned and studied histologically; however, no attempt was made to grade the amount of damage produced.

At the completion of all experiments, brains were removed and fixed in 10% formalin. Some brains were imbedded in 10% gelatin following fixation in order to preserve lesioned areas. In either case, frozen sections were cut at a thickness of 80μ and every other section saved and stained with thionin. Sagittal reconstructions were made rou-

tinely for purposes of localization. The final antero-posterior and dorso-ventral dimensions of each lesion were derived by plotting the area of the brain stem destroyed bilaterally .5 mm. from midline. Defining the lesions in this manner served to eliminate the asymmetrical portions of the lesions and allowed an estimate of the degree of destruction in the medial aspect of the brain stem which, as will be indicated later, appears essential to the ovulatory process.

RESULTS

Of the 76 rats lesioned in the midbrain on the morning of proestrus, 52 lacked tubal ova when examined approximately 24 hrs. later. Thirty-seven of these "blocked" animals were maintained until the morning of the second day after lesioning and 22 were still blocked; 15, however, had tubal ova, indicating that ovulation had merely been delayed 24 hrs. Fourteen rats which failed to ovulate by the second day following lesioning were examined at autopsy on the third day and 13 still had not ovulated. These results are summarized in Table 1.

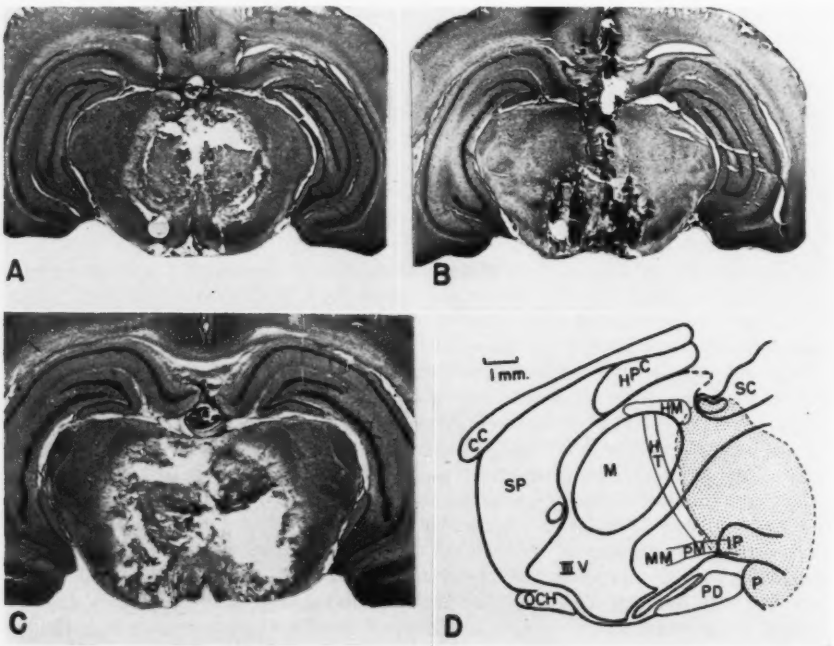

FIG. 2. A, B, C. Photomicrographs of lesions that failed to block ovulation. D. Composite diagram of the area destroyed by all lesions that failed to block ovulation (Group 2). The following abbreviations are used in this and subsequent diagrams. CC, Corpus callosum; HM, N. habenularis medialis; HPC, Hippocampus; HT, Habenulo-interpeduncular tract; IP, N. interpeducularis; M, Massa intermedia; MM, N. mammillaris medialis; OCH, Optic chiasma; P, Pons; PD, Pars distalis; PM, Peduncle of mammillary body; SC, Superior colliculus; SP, Septum pellucidum; III V, Third ventricle.

TABLE 1. SUMMARY OF THE NUMBER OF RATS EXAMINED AND FOUND BLOCKED (FAILURE TO OVULATE) ON DAYS FOLLOWING LESIONING OF THE MIDBRAIN

Time of examination	No.	Blocked	% blocked
Day 1 after lesioning	76	52	68.4
Day 2 after lesioning	37*	22	59.4
Day 3 after lesioning	14†	13	92.8

* No. of "blocked" rats carried over from day 1.
† No. of "blocked" rats carried over from day 2.

To facilitate the presentation and interpretation of the data, the 76 animals are grouped as follows:

Group 1.—Fifteen rats were autopsied and found to lack tubal ova on the morning following lesioning. Because it was subsequently found that 40.5% of the animals that are "blocked" on the first day ovulate prior to the second, no reliance could be placed on the "permanence" of the blockade produced in these rats and they were eliminated from further consideration.

Group 2.—Twenty-four rats had tubal ova when autopsied approximately 24 hrs. after lesioning. Photomicrographs of several lesions and a

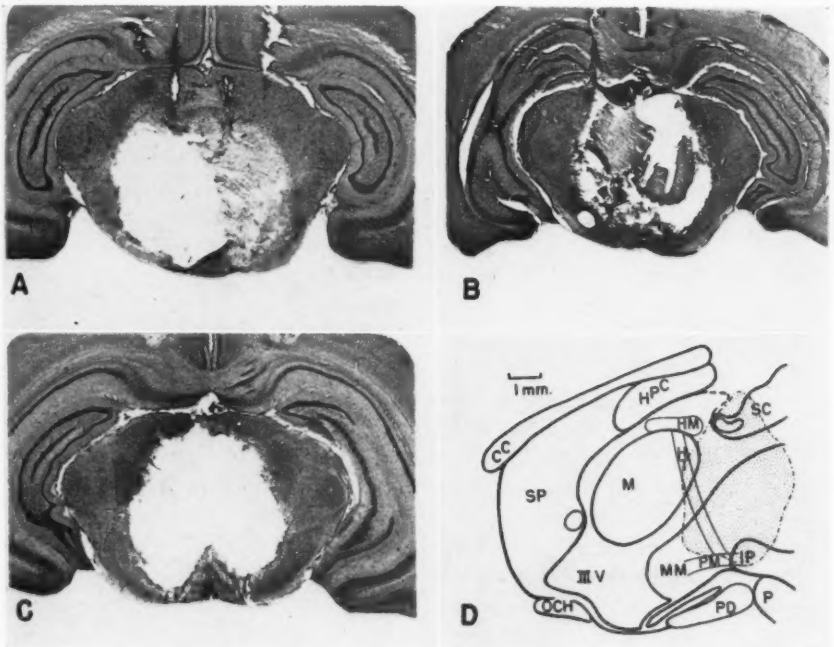

FIG. 3, A, B, C. Photomicrographs of lesions that delayed ovulation for one day. D. Composite diagram of the area destroyed by all lesions that delayed ovulation (Group 4).

composite diagram of the area of destruction produced by all of the lesions in this group of animals are shown in Figure 2.

Group 3.—Eight rats failed to ovulate prior to the termination of experimental procedures at autopsy on the second day post-lesioning.

Group 4.—Sixteen rats failed to ovulate on the night following lesioning but did ovulate subsequently. Fifteen of these animals ovulated between the first and second days after they were lesioned and one rat ovulated between the second and third days. Representative lesions and the composite group diagram are shown in Figure 3.

Group 5.—Thirteen rats were maintained for three days after being lesioned without ovulating. Because only one animal out of 14 ovulated following two consecutive days of blockade, the lesions of group 3 are considered significant and are added to those of this group. Figure 4 illustrates some of the lesions that blocked for three days and the composite diagram derived from groups 3 and 5.

To investigate the possibility of an anatomical basis for the results obtained, the composite diagrams of the various groups were compared. As shown in Figure 5A, superimposing the composite diagram from group 2 (Fig. 2) on that of groups 3 and 5 (Fig. 4) suggests that lesions which block

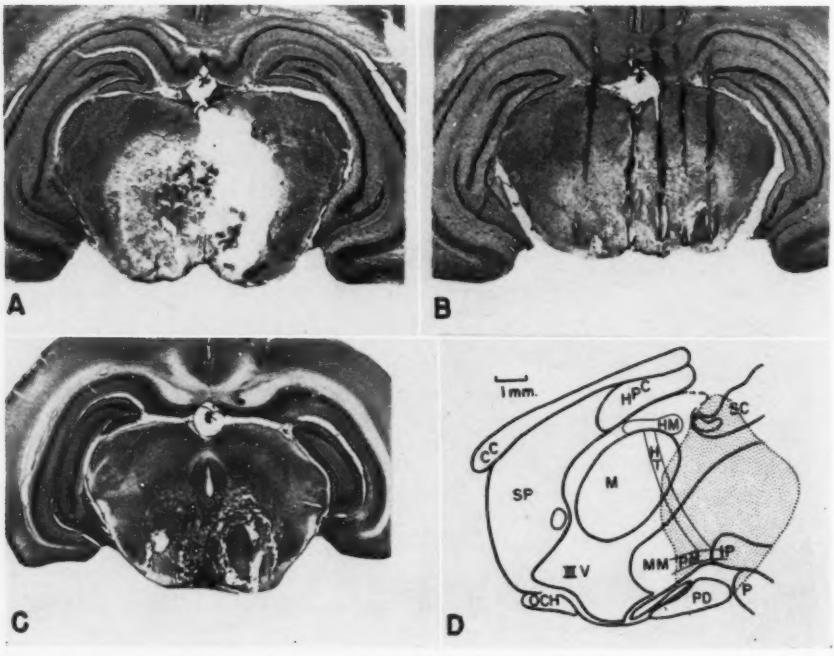

FIG. 4. A, B, C. Photomicrographs of lesions that blocked ovulation for three days. D. Composite diagram of the area destroyed by all lesions that blocked ovulation for two (Group 3) and three (Group 5) days.

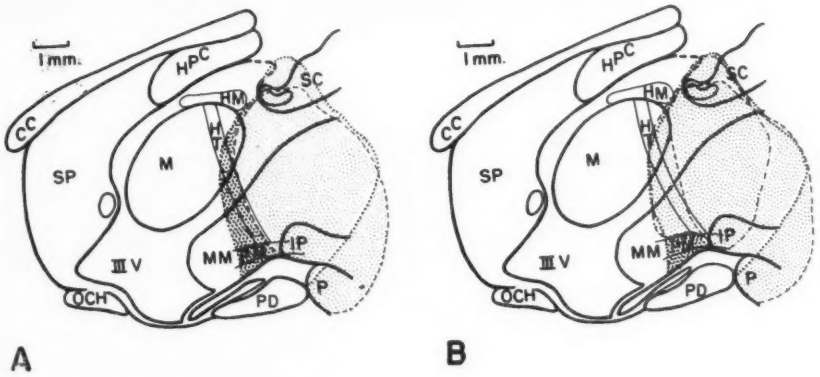

FIG. 5. Comparison of the areas destroyed in the various groups of lesioned animals. A. Result of superimposing the composite lesion diagram from animals which ovulated (Group 2, Fig. 2) on the diagram from those which were blocked for two and three days (Groups 3 and 5, Fig. 4). The darkly-stippled area is common only to those lesions that blocked ovulation for two or three days. B. Result of superimposing the composite lesion diagrams from animals which ovulated (Group 2, Fig. 2), animals which demonstrated delayed ovulation (Group 4, Fig. 3) and animals which were blocked for two and three days (Groups 3 and 5, Fig. 4). The darkly-stippled area is common only to those lesions that blocked ovulation for two or three days.

ovulation for two or three days involve an area of the brain, indicated by the dark stippling, which is not included in lesions failing to block ovulation. This area appears to have specific limits along the longitudinal axis of the brain stem: several massive lesions in groups 2 and 4, only slightly less than total tegmental transections, were ineffective while some of those in groups 3 and 5 which extended slightly more rostrad were effective. A somewhat discrete dorso-ventral position is also indicated by the finding that lesions blocked ovulation for two days or more only if they impinged upon the floor of the brain. Thus, some lesions in groups 2 and 4, although more rostrally placed than several in groups 3 and 5, failed to block for two days.

Superimposing the composite from group 4 on those of the other groups, Figure 5B, further limits the part of the brain stem which seems essential for ovulation. The darkly-stippled area is common only to the lesions which blocked ovulation for two or three days and on this basis it qualifies as the "critical area." This composite diagram also offers an explanation for the postponement of ovulation seen in group 4. Lesions in animals which were able to "escape" the initial blockade more closely encroached upon, but did not destroy, the most basal portion of the mesodiencephalic region. The transient blockades may reflect depression of adjacent structures by the acute effects of trauma and edema.

The exact anatomical difference between effective and ineffective lesions has not been sharply and precisely defined by these experiments. In fact,

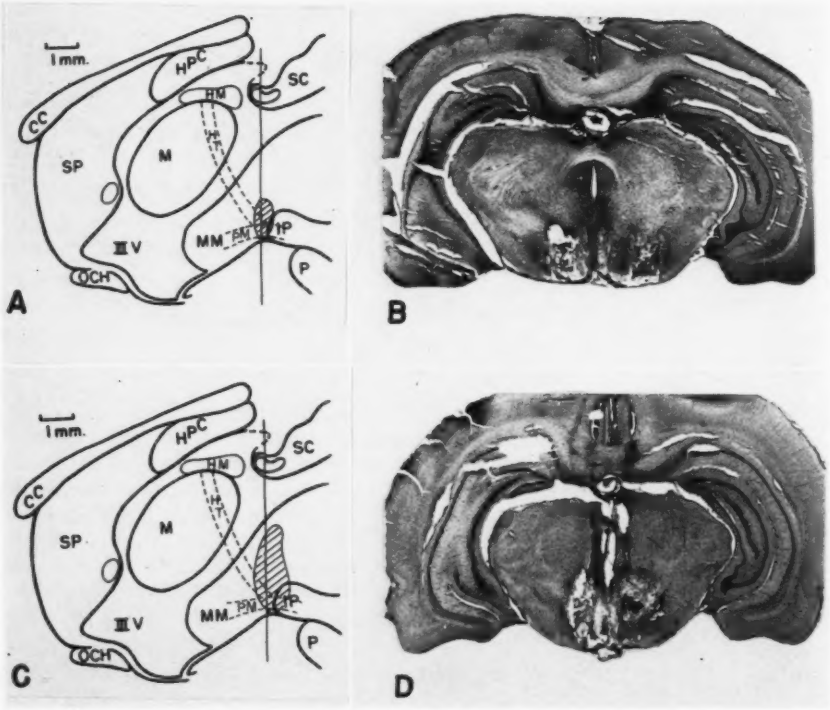

FIG. 6. Lesions that blocked ovulation for two and three days. A, C. Sagittal reconstructions of two lesions which blocked ovulation for two and three days respectively. The diagonally-lined areas indicate the longitudinal position and extent of each lesion. B, D. Photomicrographs of the lesions shown in the sagittal reconstructions A and C. The section shown in B was taken from the level marked by the vertical line in A. The level of D is similarly indicated by the vertical line in C.

there appears to be a small area of overlap between the most rostro-basal ineffective lesions and the most caudo-basal effective lesions. Two lesions that blocked ovulation appeared to be located caudad to several which did not block. Part of the overlap, approximately .2–.4 mm., may be due to the inability to differentiate conclusively between functional and non-functional tissue at the borders of the lesions; myelinated fiber systems are especially troublesome in this respect. Nevertheless, the validity of the general localization is attested by the successful blockade produced by the relatively small lesions in Figure 6 that blocked for two and three days.

The conditions of the uteri, vaginae and the ovarian tubes as they are related to the various groups of lesioned rats are shown in Table 2. Although the uteri of animals which failed to ovulate during the night following lesioning are usually distended, this condition is not always maintained throughout the three-day observation period and an estrous-type

TABLE 2. CONDITIONS OF UTERI, VAGINAE AND OVARIAN TUBES OF THE GROUPS OF LESIONED RATS ON DAYS FOLLOWING LESIONING

			Group 1, 1-day block	Group 2, no block	Group 3, 2-day block	Group 4, ov. delay	Group 5, 3-day block
	Number of rats		15	24	8	16	13
	Uterus distended		14	0	6	16	13
24 hrs. after lesioning	Vaginal smear	proestrous	1	0	2	4	4
		transitional	11	2	5	9	9
		estrous	2	22	1	3	0
	Ampulla dilated		0	24	—	—	—
	Uterus distended		—	—	4	1	9
48 hrs. after lesioning	Vaginal smear	proestrous	—	—	2	0	0
		transitional	—	—	6	2	7
		estrous	—	—	0	14	6
	Ampulla dilated		—	—	6	15	0
	Uterus distended		—	—	—	0	8
72 hrs. after lesioning	Vaginal smear	proestrous	—	—	—	0	0
		transitional	—	—	—	1	10
		estrous	—	—	—	0	3
	Ampulla dilated		—	—	—	1	0

on days two or three did not necessarily indicate ovulation. Dilatation of the ampullae of the ovarian tubes is a useful and reliable criterion for the presence of ova, but is difficult to observe without the removal of an ovary. Vaginal smears that are transitional between those characteristically observed at the proestrous and estrous stages of the cycle are fairly indicative of blockade and are useful in combination with other criteria.

Efforts to find a behavioral change specific for an ovulation-blocking midbrain lesion have been unsuccessful. Rats with rather restricted lesions in the "critical area" show little of the exploratory behavior characteristic of a normal rat and are somewhat lethargic; however, they show the righting reflex, are easily aroused by handling and respond readily and appropriately to nociceptive stimuli. The lesions shown in Figures 6A and 6C blocked ovulation without being associated with grossly observable behavioral deficits. Animals with more extensive destruction of the midbrain tegmentum are usually comatose and many are unresponsive to several types of stimuli and resemble a "reticular" preparation as described by Lindsley *et al.* (10). Such a condition, however, does not insure blockade of ovulation.

Deliberate lesioning of the pituitary on the morning of proestrus, without involvement of the midbrain, failed to block ovulation in eight rats In each case the pituitaries were examined under a dissecting microscope and found to be damaged extensively. Two rats failed to ovulate following

these lesioning procedures; however, examination revealed extension of the lesion to the overlying midbrain.

DISCUSSION

The results obtained in these experiments suggest that in the rat a restricted portion of the rostral midbrain is essential to the release of pituitary ovulating hormone. Although the size and nature of the lesions preclude discrete localization, it seems that the "critical area" is situated basally in the mesodiencephalic junction. Large lesions which almost completely transect the tegmentum at or caudal to the level of the interpeduncular nucleus are compatible with ovulation, lesions rostral to this plane block ovulation only if they extend to the most basal structures.

Massive destruction in the midbrain might be expected to block non-specifically a finely integrated process such as neural activation of the adenohypophysis. However, this does not seem to be the case: extremely large lesions and the drastic conditions with which they are associated do not block unless certain spatial requirements are fulfilled. The specificity of the blockade produced is further supported by the effectiveness of relatively small lesions. The occurrence of ovulation in spite of acute and extensive destruction of the pars distalis suggests that the blockade is not the result of direct depression of the hypophysis by non-specific trauma.

It appears that the major part of the brain stem caudal to the posterior hypothalamus is non-essential to ovulation. It is especially interesting that most of the midbrain reticular formation and some of its projections into the diencephalon are included in the apparently non-essential portion. The occurrence of ovulation in rats made somnolent and unresponsive to nociceptive stimuli by massive midbrain destruction suggests that mechanisms involved in behavioral arousal are not essential to the release of pituitary ovulating hormone. From the results obtained in these experiments and on basis of the effects of certain drugs on ovulatory and EEG arousal mechanisms (2, 4), it appears that blockade of ovulation correlates much better with drug-induced elimination of EEG arousal than with loss of behavioral arousal. The possibility exists that elements of the "reticular activating system" concerned with the EEG changes characteristic of arousal may play a critical role in the neural events leading to ovulation. The separation of the mechanisms underlying EEG and behavioral alerting responses implied by this suggestion is consistent with the dissociation seen with certain pharmacological agents: suppression of EEG arousal with atropine is compatible with behavioral arousal (11) and, conversely, physostigmine produces EEG arousal with no behavioral counterpart (12). Since cholinergic and adrenergic blocking agents can prevent ovulation in the rat (7), it is interesting that both cholinergic (13) and adrenergic (14) systems have been implicated in mechanisms of EEG arousal. Although there is no general agreement as to precise localization, a mesodiencephalic

locus has been postulated both for the cholinergic (13) and adrenergic (15) systems.

The suggested elimination of the bulk of the midbrain as an essential substrate for ovulatory mechanisms in the rat has narrowed the prospective list of "critical structures" considerably; however, the specific structure(s) involved and the role subserved are still obscure.

The structure of first choice because of its position relative to effective lesions is the mammillary peduncle. However, if the integrity of this fiber tract is necessary for ovulation, its entire longitudinal extent is not equipotential in this regard: ovulation was blocked for two or more days only by lesions that involved a site approximately .2 mm. rostral to the interpeduncular nucleus. Bilateral destruction caudad to this level was ineffective.

The mammillary peduncle is an ascending pathway (16, 17, 18) with somewhat vague origins. Investigators in the past have inferred that some fibers pass from the medial lemniscus, and additional contributions have been suggested because the tract becomes markedly enlarged just caudal to the mammillary bodies (19). Papez (20, 21) has suggested that some fibers originate from the nucleus of the mammillary peduncle, first described in mammals as the Nucleus opticus tegmenti by Tsai (22). This nucleus takes the form of an elongate cell group situated dorso-lateral to the tract. Papez also describes some fibers from the medial border of substantia nigra (21, 23). Fox (24) finds that most of the fibers arise from the ventral tegmental nucleus in the cat, not from the medial lemniscus, and also includes the nucleus of the mammillary peduncle as a potential contributor. Guillery (25) has recently investigated the mammillary peduncle of the rat using the Nauta and Gyax stain, and he concludes that fibers originate from the dorsal, and probably the deep, tegmental nuclei and possibly from the nucleus of the mammillary peduncle. This investigator considers that the "connections" with the medial lemniscus and substantia nigra are fibers of passage and do not represent additional sites of origin.

Rostrally, the mammillary peduncle appears to end mainly in the mammillary nuclei, although some fibers have been traced into the medial forebrain bundle (19), the hypothalamus (17) and as far forward as the supraoptic nucleus (26). Guillery in his recent studies (25) finds that the majority of fibers are distributed to the mammillary nuclei and, in confirmation of the earlier work, describes components passing into the medial forebrain bundle. These ascending fibers, some reaching as far as the diagonal band, diminish in number as they pass rostrally and a few are seen to turn medially towards the medial hypothalamic nuclei.

The function of this fiber system is not known although Papez (22) lists it as one of the important afferent pathways to the hypothalamus and includes it in a system of primitive centers in the ventral thalamus which receive and relay input from various afferent systems. Clark (18) believes

that the mammillary peduncle may bear the same relation to the hypothalamus as the medial lemniscus does to the thalamus. Guillery (25) feels that it may furnish the connection between the diffuse systems of the brain stem and the mammillary bodies.

Another structure of interest in this vicinity of the brain stem is the transverse peduncular tract which is located just caudal to the mammillary bodies and lateral to the mammillary peduncle (27). Edinger (28) related the fibers in this tract to the ciliary portion of the oculomotor nerve and removal of the eyes of the rat is reported to result in its degeneration (29). Clark *et al.* (30) describe this tract as originating from the optic tract near the ventral margin of the lateral geniculate and passing obliquely across the cerebral peduncle to end in the nucleus of the mammillary peduncle. This fiber system may be the basis for Ibrahim and Shanklin's statement that the only connections of this nucleus in mammals are with the optic chiasma (31). It is extremely interesting that these projections from the visual system are described as reaching the nucleus of the mammillary peduncle at a level slightly caudal to the mammillary bodies. This portion of the peduncle is the site of the marked enlargement, noted by Gurdjian (19) and is the approximate point along the longitudinal axis of the brain stem where lesions block ovulation.

Although the substantia nigra is apparently without the intriguing connections described above for the mammillary peduncle, its medial border is involved in the effective lesions and it cannot be dismissed from consideration. In spite of its classical inclusion in motor spheres, it may contribute fibers to the mammillary peduncle (21).

The interpeduncular nucleus is located very close to the area which appears necessary for ovulation and it is difficult to assess its importance in this process. However, it seems to be slightly caudal to the "critical area" and its main afferent pathway, the habenulo-interpeduncular tract, has been interrupted without blocking the release of ovulating hormone.

In speculating on a possible role for a ventral mesodiencephalic system in the process of ovulation in the rat, it seems noteworthy that some of the structures in this region appear to have wide access to many modalities of afferent input. These structures may be involved in the projection of such information to the hypothalamus, directly or indirectly. Such a system may correspond to the extra-lemniscal afferents suggested by Green (32) which project to the pituitary via the lateral hypothalamus, septum, hippocampal formation, amygdala and hypothalamus.

A role for a rather broad sensory supply to the ovulatory mechanisms in the rat is indicated by the fact that light (33, 34), temperature (35) and probably many other environmental factors with a 24 hr. rhythmicity can control the estrous cycle. However, the implication that the lesions in these studies block ovulation because of interference with sensory projections to the hypothalamus introduces complications. Ovulatory mech-

anisms are rather well insulated from the immediate and direct influence of the environment because rats blinded or placed in continual darkness, under conditions of constant temperature, retain the rhythm established under the preceding lighting conditions (34), and the reversal of the estrous rhythm by reversed day-night lighting requires about 10 days (33). The above data plus the demonstration of 24 hr. rhythmicity in the rat (9) suggest the presence of a "neural clock" which, if not intrinsic, at least possesses considerable inertia, and it is doubtful that the acute removal or manipulation of a single or several modalities of sensory input could prevent ovulation. The prompt and immediate blockade produced by the midbrain lesions may indicate a more intimate and direct relationship with ovulating mechanisms, a relationship that could be afforded by some type of "activating system."

Although the reticular formation and more caudal structures within the midbrain do not appear to be essential to ovulation in the rat, these structures may well play a critical role in neuroendocrine phenomena that rely on afferent pathways ascending from spinal levels such as ovulation in reflexly ovulating forms and the release of luteotrophin in the rat following coitus. In this regard, Anderson *et al.* (36) have proposed that transection of the brain stem at the mesencephalic level in dogs permanently blocks the release of ACTH to several kinds of stress and postulate a mesencephalic hypothalamico-pituitary activating system. In their consideration of anatomical connections from the midbrain to the hypothalamus, the authors refer to pathways proposed by Nauta and Kuypers. The mammillary peduncle and the dorsal longitudinal fasciculus are discussed as afferent systems possibly involved in the control of pituitary functions. Sayers and Sayers have reported inhibition of stress-induced ACTH release in rats following high pontine transection (37).

Although these data are suggestive, conclusions as to the role of the midbrain in the rat estrous cycle will have to await further study. Chronic lesions and midbrain stimulation experiments may be of some aid in relating this region of the brain stem to the classically-implicated hypothalamic areas and may offer some clues as to the specific parameters affected.

Acknowledgments

The author wishes to express his gratitude to Professor C. H. Sawyer for the generous guidance and counsel extended throughout the course of these experiments. Also, many thanks go to Cora Rucker and Arlene Koithan for their help with the histological preparations and to Charles Bridgman for his assistance with the illustrations.

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THE PERIPHERAL ACTION OF TOLBUTAMIDE IN DOMESTIC FOWL[1]

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ABSTRACT

The effect of Tolbutamide was studied in chickens which had been functionally hepatectomized or both depancreatized and hepatectomized. The effects of simultaneous injection of Orinase and insulin on blood glucose, NPN, and inorganic phosphate in the intact fowl were also investigated.

Hepatectomized fowl responded to 10 mg. Orinase/kg. body weight; the degree and pattern of response was almost identical with that of intact fowl of the same age. Hepatectomized birds proved to be hypersensitive to insulin, since an insulin dose that produced the same degree of hypoglycemia as Orinase in intact birds had a much more profound and prolonged hypoglycemic effect in hepatectomized birds. Birds which were depancreatized and later functionally hepatectomized also responded with hypoglycemia to Tolbutamide injection. Unless there is an extrapancreatic and extrahepatic source of insulin in the chicken, it is unlikely that the action of Orinase involves stimulation of insulin formation and/or secretion.

Intensification of insulin activity by Orinase was demonstrated in intact birds which were injected with the two drugs simultaneously. Both blood glucose and NPN were depressed to a greater degree than when either insulin or Orinase was injected alone. In contrast to insulin, Orinase appears to be ineffective in decreasing blood inorganic phosphorus in the fowl. The action of Orinase, therefore, appears to be mediated through the peripheral tissues.

CONSIDERABLE evidence is available that in certain species the hypoglycemic action of the sulfonylureas is mediated through the insulin-secreting cells of the pancreas (1, 2, 3, 4). In these species, intact beta cells must be present for 1-butyl-3-sulfanilylurea (Carbutamide) and 1-butyl-3-p-tolylsulfonylurea (Tolbutamide, Orinase) to exert their immediate hypoglycemic effects. Whether these agents increase the capacity of the beta cells to form insulin, or whether they act merely to release it, has not been established. In any event, the role of mammalian peripheral tissues in the hypoglycemic response to these agents appears to be either nonexistent or secondary to the direct action on the pancreas.

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Recently the domestic fowl was shown to be sensitive to Orinase; it may, in fact, be even more sensitive than mammals (5). Orinase may act through different mechanisms in birds and mammals, however, since neither total pancreatectomy nor enterectomy in the duck altered the response to this agent (6). These observations suggest either that insulin production is not restricted to the pancreas and/or gut in chickens and ducks or that Tolbutamide acts in these birds primarily through its effect on peripheral tissues. The studies presented in this paper indicate that the avian response to Orinase administration is regulated primarily through extrapancreatic and extrahepatic mechanisms.

METHODS

All birds employed in these studies were crosses of Single-Comb White Leghorns and New Hampshires. The birds were 10–12 weeks old and were maintained on a standard complete chicken mash until the time of experimentation. In all studies food was removed six hours prior to obtaining the first blood sample. During observation periods, all birds were immobilized by tying their legs with heavy cord. No anesthetic was used and birds previously subjected to surgery evidenced no signs of the earlier narcosis.

Blood samples were drawn by cardiac puncture. Where blood glucose was the only analysis to be made, 0.1 cc. blood was drawn. If more than one analysis was desired, 1.0 cc. blood was drawn. These methods and sampling rates have been shown to have no effect on control values of various blood constituents (5). Blood sugar determinations were made by Nelson's modification of the Somogyi method (7), non-protein nitrogen by the standard micro-Kjeldahl technique, and inorganic phosphorus by the method of Sumner (8).

Two series of birds were prepared surgically for studying the peripheral effects of Orinase: Series I birds were hepatectomized, and Series II birds were pancreatectomized and hepatectomized. An additional group (Series III) was used for studying interactions of insulin and Orinase in the intact bird. Functional hepatectomy was performed according to the method of Ranney *et al.* (9). The presence of the coccygomesenteric vessel, which connects the right hepatic portal vein with the afferent renal veins in the fowl, makes possible a naturally occurring Eck fistula. By properly placing and tying two ligatures, blood from the intestines is diverted through this anastomosis and through the kidney to the vena cava. Ligation of the hepatic arteries as well as the portal veins shuts off at least 99% of the total afferent blood flow to the liver of the bird (9). In Series I the loose ligatures were exteriorized and the birds allowed to recover 7 to 9 days before use. Instantaneous functional hepatectomy in the unanesthetized bird was then accomplished by tightening the exposed ligatures and thereby occluding all afferent vascular pathways leading to the liver. In Series II pancreatectomy was performed by using gauze pads to scrape away all pancreatic tissue. Extreme care was taken to maintain intact the network of vessels suspended in the duodenal loop and to keep hemorrhage to a minimum. Immediately after removal of the pancreas the loose ligatures were placed for subsequent hepatectomy. The bird was then allowed to recover from the Nembutal anesthesia; it was usually on its feet and drinking water within 1–2 hours after the operation. These doubly operated birds were used the day of surgery to avoid the severe inanition which follows pancreatectomy. Data are reported herein only from birds which proved to be totally depancreatized and/or functionally hepatectomized by proper ligature placement as determined at the time of autopsy.

Orinase[3] was dissolved in 0.9% NaCl and injected intramuscularly into the shoulder

[3] Generously supplied by C. J. O'Donovan, the Upjohn Co., Kalamazoo, Michigan. Lot No. Res. 11,584,2.

girdle at the dose level of 5 to 10 mg./kg. body weight; insulin free from hyperglycemic activity[4] was administered intramuscularly at the dose level of 0.5 or 1.0 u./kg. body weight as indicated in the following section. These particular dosages of Orinase and insulin were employed because preliminary studies demonstrated that the hypoglycemic response to 10 mg./kg. body weight of Orinase equaled that to 1.0 u./kg. body weight of insulin in the intact fowl, 10–12 weeks of age. The hypoglycemia produced by either agent was on the order of 22–26% of the initial blood glucose level.

RESULTS

Series I. Response of Hepatectomized Fowl to Orinase and Insulin

Thirty-five 12-week-old chickens of both sexes were employed in this study. The two loose ligatures were properly placed (care being taken to avoid including the main pancreatic vessels) and exteriorized, and the birds were allowed to recover for seven to nine days. The birds were then divided into four groups and treated as follows: 7 received 2 cc. saline, the ligatures remaining loose; 10 received the same saline dose after the liga-

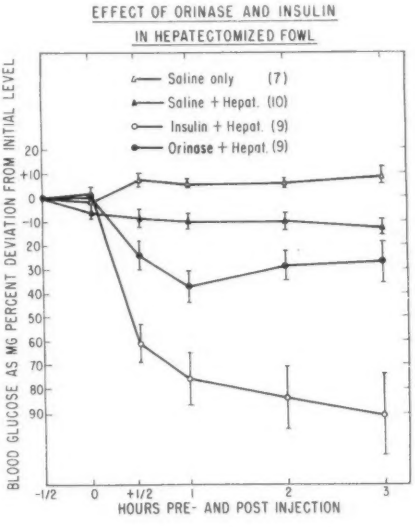

FIG. 1. The effects of Orinase and insulin in functionally hepatectomized fowl. Immediately after the first blood sample was taken the hepatectomy ligatures were tied. Thirty minutes later (at time zero) another blood sample was taken and the birds were injected as indicated. ( ) indicates number of birds per group. Vertical bars indicate standard errors of the mean.

tures were tied; 9 received 1.0 u. insulin (free of HGF) per kilogram body weight after ligatures were tied, and 9 received 10 mg. Orinase per kilogram body weight after ligatures were tied.

Blood glucose values of hepatectomized fowl treated with either Orinase or insulin are presented in Figure 1. Tying off the hepatic vessels to cause a functional hepatectomy caused the blood glucose to decrease as much as 13 mg. %. The differences between the two saline control groups are

[4] Generously supplied by W. L. Kirtley, Eli Lilly Co., Indianapolis, Indiana. Lot No. W-3375.

significant at the 5% level at every point throughout the three-hour observation period. Also, it is obvious that hepatectomized fowl responded rapidly to Orinase administration, the blood sugar level decreasing from the initial level of 203 mg.% to a level of 166 mg.% within one hour. Comparatively, however, the response to 1.0 unit insulin/kg. body weight was much more profound, for the mean blood glucose level decreased from a pre-injection value of 201 mg.% to 109 mg.% after three hours. This was unexpected since previous studies indicated that the dosages of both Orinase and insulin employed in this series decreased the blood glucose of intact fowl to the same extent.

Series II. The Effect of Orinase in the Depancreatized-Hepatectomized Fowl

It has been demonstrated that totally depancreatized as well as completly enterectomized ducks respond well to Orinase administration (6). It

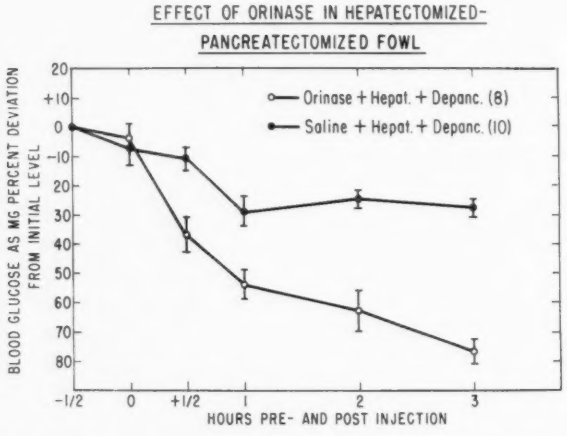

FIG. 2. Effect of Orinase in hepatectomized, depancreatized fowl. The time sequence of tying the hepatectomy ligatures and taking blood samples was identical to Series I. ( ) indicates number of birds per group. Vertical bars indicate standard errors of the mean.

appeared pertinent to determine if chickens can respond to Orinase in the absence of both the liver and the pancreas.

Eighteen female chickens, 12 weeks of age, were depancreatized. Subsequently, while the birds were still under anesthesia, the two hepatectomy ligatures were loosely placed in position, and the birds sutured and allowed to recover from the effects of the anesthetic. When the birds were on their feet, taking water *ad libitum*, and had fasted a total of 6 hours, they were functionally hepatectomized. Half an hour after the hepatectomy ligatures were tied, 10 of the birds received 2.0 cc. of physiological saline and the other 8 received 10 mg. of Orinase per kilogram body weight. Al-

though it has been reported to be very resistant to the diabetogenic effect of alloxan, the bird does respond to pancreatectomy with a moderate transitory hyperglycemia which usually lasts less than seven days (10). This hyperglycemic effect was observed here even in the absence of a functional liver since these doubly operated chickens had initial blood glucose levels (i.e., 1 to 3 hours after pancreatectomy) ranging from 251–302 mg.%.

Blood glucose values of the doubly operated birds are presented in Figure 2; the Orinase-treated group responded with a sharp decrease in blood glucose. The initial mean blood glucose level was 262 mg.% (as the result of pancreatectomy) and decreased to 185 mg.% at the end of the three-hour observation period. Blood glucose levels of the similarly operated but saline-treated controls meanwhile decreased from 270 mg.% to 242 mg.% during the same period.

Series III. Effect of Simultaneous Injection of Orinase and Insulin on Blood Glucose, Non-Protein Nitrogen and Inorganic Phosphorus of Intact Birds

The results of Series I and II suggest that the site of action of Orinase is in the peripheral tissues. To test this concept further, 38 intact male birds, 10 weeks of age, were fasted six hours, divided into five groups, and injected as follows: six birds received 3 cc. saline, eight were injected with 10 mg. Orinase/kg. body weight; seven were injected with 1.0 U. insulin/kg. body weight; nine were injected with 10 mg. Orinase and 1.0 U. insulin/kg. body weight; and eight were injected with 5 mg. Orinase and 0.5 U. insulin/kg. body weight. Since the dosages of Orinase and insulin employed here were of equal hypoglycemic potential, the possibility of observing an augmentation of the effect of the two agents was increased. The saline control values for blood glucose and non-protein nitrogen were initially 200 ± 1.6 mg.% and 41 ± 0.7 mg.% and throughout the four hours of observation did not fluctuate by more than 6 mg.% and 2 mg.% respectively. For clarity these data were omitted in Figure 3. No inorganic phosphorus analyses were made at the lower Orinase and insulin dosage level.

The augmented response by intact birds doubly treated with Orinase and insulin is apparent in Figure 3. Not only did the addition of 10 mg. of Orinase to 1.0 unit of insulin/kg. cause an expected greater hypoglycemic response, but the reduction of these dose levels by 50% also brought about a greater decrease in blood sugar than the injection of either Orinase or insulin alone. Similarly, the same increased response is demonstrated in the blood NPN data of both groups which were doubly treated. The inorganic phosphorus moiety responded rapidly to exogenous insulin and after an initial decrease rebounded to supernormal levels in chickens. Orinase does not appear to affect this phosphorus fraction, and doubly treated fowl do not show an additive effect as is seen in the blood glucose and NPN data.

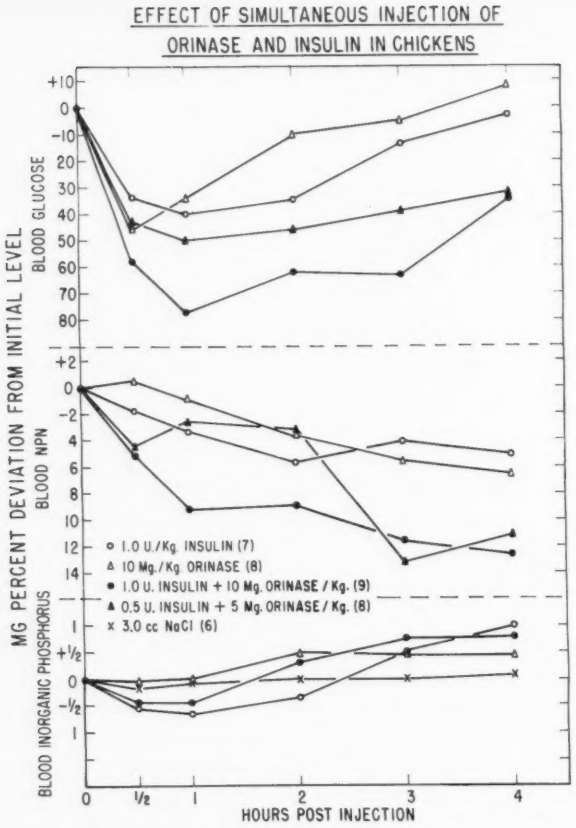

FIG. 3. Effects of simultaneous injection of Orinase and insulin into intact 10-week-old chickens. For all groups the initial range of blood glucose was 198–211 mg.%; blood NPN, 41–48 mg.%; and inorganic phosphate, 4.91–5.83 mg.%.

DISCUSSION

The observation that depancreatized fowl exhibit only a temporary hyperglycemia (lasting several days) has prompted many workers to adopt the hypothesis that the pancreas is not the major site of insulin production in the bird. Similarly the recent evidence that depancreatized ducks respond to Orinase administration as readily as do intact control ducks indicates either alternative mechanisms of Orinase action or the existence of an additional site, or sites, of insulin formation. Mirsky and Gitelson (6) have found that liver extracts from ducks exert no hypoglycemic effect when injected into recipients.

The data from Series I indicate that Orinase action is not mediated through the liver. These findings are in accord with those reported for the dog and rat (11). The response of hepatectomized fowl to 10 mg.

Orinase/kg. body weight is almost identical to the hypoglycemia reported for the intact bird of the same age receiving the identical dose (5). The rapid response of the functionally hepatectomized bird to insulin, also demonstrated in Series I, is in accord with many studies in mammals. However, where the blood glucose level of the Tolbutamide-treated birds decreased to the lowest point one hour after injection, the insulin-treated fowl showed a continual decrease throughout the entire observation period. The resulting profound hypoglycemia suggests that the liver plays an important role in insulin degradation in the bird. With the liver removed from the circulation, the activity of insulin was intensified and could act over a longer period of time. The lack of any effect of hepatectomy in the response of birds to Orinase, by contrast, suggests that Tolbutamide both acts and is destroyed in the peripheral tissues.

The results of Series II demonstrate the hypoglycemic action of Orinase in birds which have been both depancreatized and functionally hepatectomized. In these doubly operated birds, the blood sugar decreased approximately twice as much as in those which were hepatectomized only. Also, the decrease in the controls' blood glucose levels was greater than in the hepatectomized controls of Series I. These differences might be due to the fact that the doubly operated birds were used on the day that surgery was performed and thus were suffering somewhat from blood loss and subsequent hemodilution as a result of surgical trauma. Mann and Bollman (12) have demonstrated that hepatectomy performed after pancreatectomy in dogs causes a gradual decrease in the diabetic blood glucose levels. The control data of Series II agree with these findings.

The observations that depancreatized ducks, enterectomized ducks, and alloxanized chickens respond to Orinase administration (6) are complemented by the data of Series I and II. The results of Series III demonstrated the additive nature of responses, both of glucose and of NPN, to simultaneous treatment with Orinase and insulin (Fig. 3). This increased response resulted in an accentuated hypoglycemia and protein-sparing action even when dosages were reduced to half the original levels. These results are in contrast with findings in the rat (13) where Orinase-pretreated animals were not hypersensitive to subsequent insulin administration. In all studies, preliminary as well as those reported herein, where insulin-treated fowl are compared with Orinase-treated fowl, the blood glucose level of the latter decreased more rapidly than those of the former. Generally, hypoglycemia due to Orinase administration reaches its extreme 30–60 minutes after injection, whereas insulin required 60–120 minutes to produce the same degree of hypoglycemia.

The inorganic phosphate data of the Series III birds are consistent with the results of many mammalian studies. Blood inorganic phosphate was decreased in fowl as it has been in mammals injected with insulin. Orinase, like Carbutamide, which has been shown to have no effect on this phos-

phate moiety of the dog (14), similarly had no effect in the fowl. The decrease in blood phosphate of the doubly treated group of birds was no greater than that of those treated with insulin alone and was probably due only to the insulin portion of the treatment.

If the beta cell stimulation hypothesis of Orinase action, currently held for mammals and certain amphibia, is to be applied to the domestic fowl also, it must be concluded that there exists not only an extrapancreatic but also an extrahepatic source of insulin in the bird. The intestinal tract, as a possible site of Tolbutamide action, has been investigated and ruled out by Mirsky and Gitelson. These findings suggest an alternative site of insulin formation or an alternative mechanism of Orinase action, possibly in the peripheral tissues. The results presented here favor the hypothesis that Orinase action is mediated through the periphery but that it is not an insulin-like agent itself nor does it involve the stimulation of insulin formation and/or secretion in the fowl.

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CAPACITATION OF RABBIT SPERMATOZOA IN THE UTERUS WITH SPECIAL REFERENCE TO THE REPRODUCTIVE PHASES OF THE FEMALE[1]

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ABSTRACT

The capacitation of sperm (spermatozoa undergo a certain physiological change in the female tract before they are capable of fertilization) was investigated by injection of sperm into the uterus of rabbits at various reproductive phases or rabbits treated with gonadotrophin, estrogen or progesterone. Twelve hours later the injected sperm were recovered and then deposited into the top of the fallopian tubes of another rabbit that had ovulated recently. This was done to determine whether sperm so treated could fertilize the ova. Although capacitation was achieved in the uterus of immature and ovariectomized rabbits with or without the treatment of gonadotrophin or estrogen, it could not be achieved in the uterus of pseudopregnant rabbit with or without the treatment of gonadotrophin or estrogen or in the uterus of immature, ovariectomized, or estrous rabbits treated with progesterone. The inhibition of capacitation of sperm in the pseudopregnant uterus or in the uterus of rabbits treated with progesterone was not due to the poor motility of sperm, the large number of sperm, the concentration of white cells, or the possible bacterial infection of the endometrium. However, when sperm were injected into the fallopian tubes of pseudopregnant rabbits and then recovered and deposited into the tubes of another rabbit fertilization occurred. It seems that capacitation of sperm was inhibited in the uterus under the influence of progesterone but it was not so affected in the fallopian tubes.

BY DEPOSITION of ejaculated sperm into the fallopian tubes of rabbits at various times before or after ovulation and by deposition of sperm recovered from the uterus at various times after mating it has been shown by Chang (3, 4) that fertilization of ova can occur only when sperm have been in either the tube or the uterus for about 6 hours. This phenomenon was also observed in the rat as well as the rabbit and termed as capacitation of sperm by Austin (1, 2). Although injection of semen into the uterus of estrous rabbits by surgical means for 5 hours achieves capacitation (3) it is not known whether sperm can be capacitated in the uterus of an animal at different reproductive phases. This paper reports a series of

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tests to determine whether rabbit sperm can be capacitated in the uterus of immature, ovariectomized and pseudopregnant rabbits or in the uterus of rabbits at various reproductive phases under the treatment of gonadotrophin, estrogen or progesterone.

ANIMALS AND METHODS

Adult non-pregnant female rabbits of mixed breeds were purchased from local farms. They were kept in separate cages in the laboratory for about 4 weeks before use. Estrus was ascertained by the watery and dark red color of the vulva, by several fast spasmodic reflexes of the pelvis when the vulva was touched by a finger, and by the presence of large follicles in the ovaries at operation or at autopsy. Pseudopregnancy was induced by an intravenous injection of sheep pituitary extract which invariably leads to ovulation. Ovariectomy was performed through a flank incision under Nembutal anesthesia and in the majority of cases the fallopian tubes were also removed. The animals were used from 19–80 days after ovariectomy. Immature rabbits were young rabbits, 2 to 3 months old, either purchased from local farms or raised in the laboratory.

For the gonadotrophin treatment 5–7 mg. (28–40 I.U.) of sheep pituitary extract, a dose adequate to induce ovulation, was intravenously administered 4 hours before the injection of semen into the uterus. For the estrogen treatment, a total dose of 1.5 mg. of estradiol in sesame oil was subcutaneously administered, within 2 or 3 days, 12 hours before the injection of semen. For progesterone treatment, 25 mg. of macrocrystalline progesterone was subcutaneously administered to an adult animal, or 12.5 mg. to an immature animal, 3–4 days before the injection of semen into the uterus.

Semen of 3 fertile male rabbits reared in the laboratory was collected regularly at an interval of 6–8 days by means of an artificial vagina, but only a single ejaculate of one male was used at a time. About 0.1 ml. of penicillin in saline (1,000 units) was added to an ejaculate (0.5 to 1 ml.) as an antiseptic measure, and the sample was taken up with a tuberculin syringe. A female rabbit was anesthetized by Nembutal and a midline incision near to the vagina was made. When the uteri were exposed, 0.15 to .25 ml. of semen was injected into the lumen of one uterus by means of a 21 gauge needle.

The same semen sample was usually injected into only a single uterine horn of 2 or 3 rabbits, for instance, one estrous, one pseudopregnant, or one ovariectomized, in the evening from 9 to 10 P.M. Immediately after operation another 2 to 4 rabbits were injected with pituitary extract intravenously in order to induce ovulation for the deposition of sperm recovered from uterus. Next morning from 9 to 10 A.M. that is, 12 hours after the injection of semen into the uterus, the uterus that had been injected with sperm was carefully removed and flushed with 5 ml. of warm saline. In order to concentrate spermatozoa for deposition the uterine washing was centrifuged at 3,200 R.P.M. for 20 minutes. Most of the supernatant fluid was then carefully removed, but 0.3 to 0.5 ml. of remaining fluid was thoroughly mixed with sperm at the bottom of the tube. The motility of sperm in the centrifuged uterine washing was examined under a low power microscope and was recorded with symbols 0, 1, 2, 3; 0 denotes no progressive motility, 3 the best motility.

A flank incision was made on the rabbits that had been injected intravenously with pituitary extract at 9–10 P.M. and the fallopian tube was exposed. (Since rabbits ovulate about 10 hrs. after an intravenous injection of pituitary extract, at this time, 9–10 A.M., the ova in the fallopian tube were about 2 to 3 hours post-ovulatory.) Then about 0.04 to 0.05 ml. of centrifuged uterine sperm suspension in a glass pipette, 1 mm. in diameter, was deposited into the top of the fallopian tube through the infundibulum. In order to control the variations in sperm quality and the viability of ova, sperm recovered from the uterus of an estrous rabbit or from a rabbit treated with estrogen were deposited into the left tube and those recovered from the uterus of a pseudopregnant rabbit or

from a rabbit treated with progesterone were deposited into the right tube of the same rabbit. After deposition, the concentration of sperm and white cells in the sperm sample were estimated by means of haemocytometers, and the number of sperm deposited into each tube was calculated. One day later, the fallopian tube was removed and flushed with saline. The ova thus recovered were mounted *in toto* on a slide and examined under a high magnification microscope before, after fixation, and after staining (5). The ova would be at a 4–8 cell stage if fertilized, or at the second maturation spindle stage with only one polar body if unfertilized. The number of sperm on the zona pellucida of each ovum was counted. If the number was more than one hundred, it was noted as a hundred.

RESULTS

Table 1 presents data obtained when rabbit semen was injected into the uteri of pseudopregnant or of estrous rabbits for 12 hours and then deposited into the fallopian tube of another rabbit that had ovulated very recently. From Table 1 it is quite obvious that essentially no ovum was fertilized if the deposited sperm were incubated in the uterus of 6–14 days pseudopregnant rabbits with or without gonadotrophin or estrogen treatment. There were, however, 63% of fertilized ova obtained when the deposited sperm had been incubated in the uterus of an estrous rabbit for 12 hours. In contrast, only 2.1% of ova were fertilized if the deposited sperm had been incubated in the uterus of an estrous rabbit that had been treated with progesterone. It seems therefore that treatment of pseudopregnant rabbits with either gonadotrophin or estrogen did not improve their ability to capacitate sperm, but treatment of estrous rabbits with progesterone did inhibit their function of sperm capacitation.

The number of sperm recovered from the uterus of a pseudopregnant rabbit or of estrous rabbit treated with progesterone in general was more than that recovered from the uterus of an estrous rabbit. This is shown by a higher number of sperm in a definite volume that was deposited into each tube as presented in Table 1. The average motility grade of sperm recovered from the pseudopregnant uterus was only slightly lower than that recovered from the estrous uterus, and yet the fertilizing capacity of sperm recovered from the pseudopregnant uterus was practically nil (0–2.4%) as compared with that recovered from the estrous uterus (63%). In this respect, the motility of sperm recovered from the uteri of estrous rabbits treated with progesterone was just as good as that of sperm recovered from estrous rabbits, but there was a great difference in their fertilizing capacity (2.1% vs. 63%). The proportion of sperm heads was higher in the samples obtained from the uterus of the pseudopregnant rabbit without treatment or with the treatment of estrogen (31–34%) than in those samples obtained from the uterus of pseudopregnant rabbits treated with gonadotrophin (4%) or from the uterus of the estrous rabbit without treatment (3.3%) or of the estrous rabbit treated with progesterone (4%). Only a very few ova had no sperm on the zona pellucida, and most of them had a great many sperm on their zona pellucida whether fertilized or unfertilized.

Table 2 presents data obtained when rabbit semen was injected into the

TABLE 1. CAPACITATION OF SPERM IN THE UTERUS OF PSEUDOPREGNANT AND ESTROUS RABBITS

Semen injected into the uterus	No. of rabbits injected with semen	No. of rabbits deposited with sperm	Sperm quality			Avg. conc. of white cells/mm.[3]	No. of ova examined			Avg. no. of sperm on zona	
			Avg. no. and range deposited (in M.)	Motility grade	Avg. and range of sperm heads		Total	Fertilized	% of Fertilization	Fertilized ova	Unfert. ova
6–14 days pseudopregnant rabbits No treatment	6	5B+10 U[1]	3.81 (.27–7.0)	2 (1–3)	31% (5–60)	9,830 (50–21,100)	84	1+1?	2.4%	9 (1–17)	36.2 (0–100)
Gonadotrophin treated	3	10 U	3.93 (1.8–6.2)	1.8 (1–3)	4% (2–7)	6,230 (5,350–7,100)	47	0	0	—	48.5 (0–100)
Estrogen treated	3	10 U	6.42 (.04–12.5)	1.8 (0–3)	34% (10–70)	2,820 (2,100–3,250)	58	0	0	—	37.5 (0–100)
Estrous rabbits No treatment	6	5B+12 U	.21 (.02–1.2)	2.7 (1–3)	3.3% (0–14)	4,100 (750–8,400)	95	60	63%	33 (0–100)	12.1 (0–100)
Progesterone treated	5	9 U	1.43 (.14–2.4)	3 (3)	4% (0–9)	5,730 (3,200–10,750)	47	1	2.1%	4	42.5 (3–100)

[1] 5B denotes that sperm samples of the same treatment were deposited into both tubes of five animals. 10 U denotes that sperm samples of the same treatment were deposited into one tube of ten animals and sperm samples of different treatment were deposited into the other tube.

uteri of immature or ovariectomized rabbits for 12 hours and then deposited into the fallopian tube of another rabbit that had recently ovulated. From Table 2 it is clear that capacitation of sperm can be achieved in the uterus of the immature or as ovariectomized rabbit with or without the stimulation of either gonadotrophin or estrogen. This shows that the capacitation of sperm in the uterus is not necessarily enhanced by the participation of estrogen or gonadotrophin. By treatment of immature or ovariectomized animals with progesterone, however, the capacitation of sperm in the uterus was inhibited. This is shown by the fact that only 3 to 8% of fertilized ova were obtained when the deposited sperms were recovered from the uteri of animals treated with progesterone whereas 26 to 69% of fertilized ova were obtained when the deposited sperm was recovered from the uteri of immature or ovariectomized rabbits, with or without the treatment with either gonadotrophin or estrogen. There was no obvious difference in the number of sperm recovered from the uterus as reflected by the number deposited into each tube or in the motility of sperm recovered from the uterus of these animals with or without treatment. The number of sperm recovered from immature or ovariectomized animals in general was lower than that recovered from pseudopregnant animals but not much lower than that from the estrous animals (Tables 1 and 2). There was some indication that the concentration of white cells was higher in those immature animals treated with estrogen and in those ovariectomized animals treated with estrogen or gonadotrophin. The average number of sperm on the zona pellucida, in general, was higher in the fertilized ova than in the unfertilized ova.

Throughout this study penicillin was added to semen before injection into the uterus as an antiseptic measure. In order to determine whether or not the addition of antibiotic to semen plays a part for the capacitation of sperm in the uterus, aliquots of an ejaculate, one with penicillin (2,000 units/ml. of semen) and one without penicillin were injected into the left or into the right uterus of estrous rabbits. When the sperm recovered from the penicillin-treated and untreated samples were deposited into (separate) tubes of a rabbit the proportion of fertilized ova was very much the same on the two sides (58% vs. 56%) as shown in Table 3.

As it is known that the uterus is more susceptible to infection at the luteal phase than at the follicular phase (11) and that semen contains a large number of bacteria (8) it was thought that the inhibition of the capacitation of sperm in the pseudopregnant uterus might be due to a microbial reaction. Thus penicillin (2,000 units/ml. of semen) and streptomycin (60–110 mg. streptomycin sulfate/ml. of semen) were added to the semen and then injected into the pseudopregnant uterus to determine whether or not the capacitation of sperm could be achieved in the pseudopregnant uterus. Furthermore, the bacterial contamination would be eliminated if epididymal sperm were used. Thus sperm recovered from excised cauda epididymis suspended in saline without the addition of antibiotics were in-

TABLE 2. CAPACITATION OF SPERM IN THE UTERUS OF IMMATURE AND OVARIECTOMIZED RABBITS

Semen injected into the uterus	No. of rabbits injected with semen	Sperm quality				Avg. conc. of white cells/mm.^{3}	No. of ova examined			Avg. no. of sperm on zona	
		No. of rabbits deposited with sperm[1]	Avg. no. and range of sperm deposited (in M.)	Motility grade	Avg. and range of sperm heads		Total	Fertilized	% of Fertilization	Fertilized ova	Unfert. ova
Immature rabbits No treatment	3	7	.15 (.06–.29)	2.7 (2–3)	8.3% (4–16)	17,250 (1,680–31,450)	32	11	34%	58.4 (8–100)	34.1 (3–100)
Treated with estrogen	3	7	.189 (.16–.22)	3 (3)	8.6% (4–14)	34,660 (10,750–73,200)	32	17	53%	54.5 (8–100)	41.5 (0–100)
Treated with gonadotrophin	3	6	.098 (.075–.14)	3 (3)	7.3% (0–15)	4,390 (1,750–8,750)	32	22	69%	39 (3–100)	7.7 (2–13)
Treated with progesterone	3	6	.89 (.63–1.12)	3 (3)	9.6% (1–22)	1,730 (650–2,450)	32	1	3.1%	42	33.7 (7–100)
Ovariectomized rabbits No treatment	4	11	.24 (.11–.45)	2.5 (2–3)	7.6% (5–10)	18,960 (2,650–67,550)	41	15	37%	51 (5–100)	23.6 (0–95)
Treated with estrogen	4	11	.98 (.35–1.5)	3 (3)	8.6% (3–16)	89,510 (39,200–179,500)	48	16	33%	70.5 (21–100)	48.3 (0–100)
Treated with gonadotrophin	5	9	.58 (.25–1.1)	2.9 (2–3)	3.8% (0–6)	84,520 (15,630–325,000)	42	11	26%	47.0 (3–100)	29.4 (0–100)
Treated with progesterone	5	10	1.09 (.26–1.9)	2.6 (1–3)	6.4% (1–20)	3,310 (575–6,350)	36	3	8.4%	23 (11–36)	34.4 (0–100)

[1] Sperm samples of the same treatment were deposited into one tube of each animal and sperm samples of different treatment were deposited into the other tube.

TABLE 3. CAPACITATION OF SPERM IN THE UTERUS OR IN THE FALLOPIAN TUBES UNDER VARIOUS CONDITIONS

Sperm injected into	No. of rabbits injected with sperm	No. of rabbits deposited with sperm[1]	Sperm quality			Avg. conc. of white cells/mm.[3]	No. of ova examined			Avg. no. of sperm on zona	
			Avg. and range of sperm deposited (in M.)	Motility grade	Avg. and range of sperm heads		Total	Fertilized	% of fertilization	Fertilized ova	Unfert. ova
Uterus of estrous rabbits No treatment of semen	3	6	.15 (.017–.35)	2.8 (2–3)	2.5% (2–3)	750	31	18	58%	9.4 (0–24)	4.3 (0–23)
Treatment of semen with penicillin	3	7	.21 (.07–.35)	2.7 (2–3)	2.7% (1–5)	5,560	36	20	56%	32 (0–100)	16.8 (1–45)
Uterus of pseudopregnant Semen treated with penicillin and streptomycin	5	11	1.75 (.2–4.72)	2.4 (2–3)	8.3% (4–23)	38,330 (15,750–121,000)	52	4	7.7%	31.5 (10–57)	56.1 (9–100)
Epididymal sperm used	4	10	.75 (.04–1.88)	2.7 (2–3)	11% (1–70)	6,490 (1,750–14,250)	42	4	9.5%	42.3 (20–100)	38.7 (0–100)
Uterus of 1 day pseudo-pregnant rabbits	2	4	.15 (.02–.29)	2 (2)	3.5% (1–10)	13,600	29	7	24%	8.1 (1–23)	14 (0–44)
Uterus of 19 days pseudopregnant rabbits	2	5	.23 (.15–.32)	3 (3)	4.4% (3–6)	40,010 (11,450–153,230)	23	9	39%	29.3 (7–69)	18.7 (6–61)
Tubes of 7–9 days pseudopregnant rabbits	2	7	.09	2 (2)	2% (2–3)	600	45	23	51%	26.3 (2–77)	31.2 (0–100)
Tubes of estrous rabbits	2	5	.12 (.07–.29)	1.2 (1–2)	13.8% (1–17)	840 (825–900)	27	20	74%	12.6 (0–60)	2.5 (0–6)

[1] Sperm samples of the same treatment were deposited into one tube of each rabbit and sperm samples of different treatment were deposited into the other tube.

jected into the pseudopregnant uterus. As shown in Tables 1 and 3, treatment of ejaculated semen with antibiotic or use of epididymal sperm increased the proportion of fertilized ova only slightly (7.7–9.5% vs. 0–2.4%) but did not increase it to the same extent as did the deposition of sperm recovered from estrous uterus (63%). It appears that the inhibition of capacitation of sperm in the pseudopregnant uterus is not entirely due to possible infections but is due to certain unknown reactions between spermatozoa and the progestational endometrium.

All the pseudopregnant animals used in this study were at 6 to 14 days after ovulation, at which time the development of corpora lutea is at its peak. A few tests were performed to determine whether capacitation of sperm can be achieved in the uterus before the full development of the corpora lutea, or at the end of pseudopregnancy. As shown in Table 3, the capacitation of sperm in the uterus was possible if the animals were used at 1 day or 19 days after ovulation. It seems, therefore, that the inhibition of capacitation occurs only at the peak of the progestational activity of the uterus.

It is known that following administration of gonadotrophin to induce ovulation the fertility of pseudopregnant rabbits is practically nil, although about 60% of fertilized ova were obtained when insemination was performed via the uterus instead of via the vagina (9, 10). In the present study when semen was injected into the uterus of a gonadotrophin-treated pseudopregnant rabbit, fertilized ova at pronuclear stages were invariably obtained from the tubes at the time of the removal of uterus for flushing of sperm. As it is demonstrated in the present study that capacitation of sperm cannot be achieved in the pseudopregnant uterus, it was thought that the capacitation of sperm must have been achieved in the tubes of the pseudopregnant rabbits, otherwise fertilization could not have occured. When 0.2 ml. of semen was injected into each tube of pseudopregnant or of estrous animals for 12 hours and then sperm was recovered and deposited into the tube of another animal, 51% or 74% of fertilized ova were obtained as shown in Table 3. It is quite clear, therefore, that capacitation of sperm in the fallopian tubes does occur in pseudopregnant animals.

DISCUSSION

It is shown in Tables 1, 2 and 3 that the number of sperm deposited into each tube was in general higher in the samples recovered from the pseudopregnant uterus or from the uteri of animals treated with progesterone than in those samples recovered from other animals. It was thought that the lack of fertilization in the former case might be due to an inhibitory effect when the number of sperm around the ovum was too high. This is, however, not so because (a) the ranges of sperm number deposited into each tube in the cases where fertilization occurred and in those cases where no fertilization occurred overlapped and (b) the number of sperm deposited into the tube was not low in the cases where a few fertilized ova were ob-

tained by deposition of sperm recovered from the pseudopregnant uterus or from the uterus of animals treated with progesterone.

Furthermore, it was also thought that the capacitation of sperm in the female tract might be a simple process to eliminate a large number of sperm in the uterus rather than a physiological change of spermatozoa in the female tract. Thus, freshly ejaculated sperms or epididymal sperms were suspended at various concentrations in saline or in uterine extract (obtained by recovery of saline that had been injected into the uterus for 6–12 hours) for deposition. It was also tested by incubation of ejaculated sperm or epididymal sperm at various concentrations in uterine extract for 6–12 hours at 38° C. However, when these sperm samples (the number of sperm deposited into each tube ranged from .002 to 1.15 million) were deposited into the tubes of 15 animals that had recently ovulated, only one out of 118 ova was fertilized. It seems quite evident that it is not the number of sperm around the ova that plays a part but a physiological change of sperm during their sojourn in the female tract that enables them to penetrate through the zona pellucida.

The appearance of uterine washings obtained at the recovery of sperm from the uterus varied. Some samples were white and opaque with white tissue debris and others were transparent and clear. Although white and opaque fluid was often obtained from the pseudopregnant uterus, it was also obtained from the estrous uterus or in the uterus of rabbits under treatment. This observation led us to determine the white cell concentration of each sample. As shown in Tables 1 and 2, there is no obvious correlation between the white cell concentration and the fertilizing capacity of spermatozoa. The high concentration of white cells may be correlated with estrogen stimulation, as shown in Table 2, in the immature and ovariectomized animals, or it may be due to possible infection following operation.

The motility of all sperm samples at the time of injection into the uterus was very good. The motility of sperm after a sojourn in the uterus for 12 hours and after the centrifugation of the uterine washing was also very good; progressively motile spermatozoa were observed among a great number of white and red cells in every case even though they were agglutinated. When freshly ejaculated semen was incubated alone or incubated at 38° C in saline, in saline containing serum, or in saline extracts of the estrous uterus for 6–12 hours, the spermatozoa varied from not motile, to sluggishly or actively motile, but these never had fast space-gaining activity for so long a time as those recovered from the uterus. It appears that the metabolism of sperm in the uterus is quite different from that in the seminal plasma or in various artificial media as shown by the maintenance of their motility. Furthermore, although the spermatozoa recovered from the uterus of the pseudopregnant rabbit or the rabbit treated with progesterone showed good motility, they were unable to fertilize an ovum. This indicates that the maintenance of motility and the capacitation of sperm are two different physiological reactions in the uterus.

The disappearance and disintegration (separation of sperm tail from head) of sperm in the uterus of estrous rabbits have been reported (6). The relatively large number of sperm and the relatively high proportion of sperm heads present in the pseudopregnant uterus as shown in the present study indicate that the disappearance of sperm is less severe than the disintegration of sperm in the pseudopregnant uterus. Since the capacitation of sperm was inhibited in the pseudopregnant uterus, it seems that the disintegration of sperm in the uterus is not necessarily a selective process for the capacitation of sperm.

It is expected that the biochemical constituents and the physiological activity of the uterine lumen at the follicular phase or under the influence of estrogen would be different from those at the luteal phase or under the influence of progesterone. Though we do not know exactly the physiological mechanism of the capacitation of sperm in the uterus or in the fallopian tube, it appears that the capacitation of sperm is independent of gonadotropic or estrogenic stimulation of the uterus, and that its physiological mechanism is inhibited by the progestational reaction of the endometrium under the influence of progesterone. Since the capacitation of sperm in the fallopian tubes was not inhibited at the luteal phase, it implies that either the activity of the tube is entirely independent of the influence of progesterone or that the physiological mechanism of capacitation of sperm in the tube is different from that in the uterus. As it has been shown that capacitation of sperm can be partially achieved in the uterus and then accomplished in the fallopian tube (4), it is unlikely that the physiological mechanism of capacitation in the uterus is different from that in the fallopian tubes. Moreover, it has been shown that the capacitated sperm (recovered from the uterus 12 hours after mating) can be rendered incapable of fertilization by treatment of sperm with 5% of seminal plasma (7). In this respect one wonders if the spermatozoa were actually capacitated in the pseudopregnant uterus and then rendered incapable of fertilization by certain constituents present in the progestational endometrium, or if there are some constituents present in the pseudopregnant uterus that block the mechanism of capacitation.

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PAPER ELECTROPHORETIC STUDIES ON PITUITARY EXTRACTS FROM NORMAL AND THYROIDECTOMIZED RATS[1]

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ABSTRACT

Paper electrophoretic analysis of saline extracts of rat adenohypophyseal tissue has demonstrated the presence of a protein component which disappeared following thyroidectomy or thiouracil feeding, and was restored after thyroxine administration. Since castration and adrenalectomy were without effect on this constituent, it is referred to as the "thyroid-labile" component of the adenohypophysis. Extracts of rat anterior pituitary glands which had previously been incubated *in vitro* in a medium containing lysine-2-C^{14} were subjected to paper electrophoresis. Radioactivity was detected in the region of the "thyroid-labile" component. Since the amount of radioactivity in this area was the same in extracts from normal and thyroidectomized rats it was concluded that the isotope had been incorporated into minor protein constituents with mobilities similar to that of the "thyroid-labile" component. Both growth hormone and thyrotropin were localized in the region of the "thyroid-labile" component, but differences in post-thyroidectomy disappearance rates of the hormones and the "thyroid-labile" component were incompatible with the view that the latter might be hormonal in nature. The hypothesis is advanced that the "thyroid-labile" component is related to the acidophilic cells of the adenohypophysis.

THE functional state of the thyroid gland has a marked influence on the structure and function of the adenohypophysis. Thyroidectomy results in enlargement of the pituitary gland (1–4), accompanied by hypertrophy and vacuolization of the basophils and degranulation of the acidophils of this structure (3, 5–9). Production of thyrotropin (10–12) and oxygen consumption of pituitary tissue *in vitro* (4) are also influenced by the level of thyroidal activity.

These findings suggested that further studies were in order on the influence of thyroid function on the biochemistry of the adenohypophysis. The present investigation demonstrates a striking change in the electrophoretic pattern of the soluble proteins of the rat pituitary gland which can be related specifically to the action of the thyroid hormone.

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MATERIALS AND METHODS

Paper electrophoresis of pituitary extracts

Male, Sprague-Dawley rats were subjected to thyroidectomy, castration, or adrenalectomy under ether anesthesia. The adrenalectomized rats were maintained on 0.9% saline drinking water. Some of the animals in each group were given daily injections of either 20 μg. of *dl*-thyroxine, 1 mg. of testosterone propionate, or 2 mg. of cortisone, as replacement therapy. All were injected subcutaneously for 7 days prior to killing and were killed 21 days after the operation. Unoperated rats were fed a diet of ground Purina laboratory chow containing 0.1% thiouracil. Some of these latter animals were injected with 20 μg. of *dl*-thyroxine daily for the last 10 days of the 30-day feeding period.

The rats were killed by exsanguination under nembutal anesthesia, the pituitary glands were removed, and the posterior lobes separated and discarded. Groups of 8 adenohypophyses were pooled and homogenized in 0.1 cc. of cold saline in ground-glass homogenizers of 0.3 cc. capacity (7×50 mm.). The homogenizer tubes were placed in plastic adapters fitted to the multi-speed attachment of the International refrigerated centrifuge. The samples were centrifuged in the cold (4° C) for one hour at 15,000×g. A micropipette was used to apply 0.02 cc. of the supernate to buffer-moistened strips of Whatman No. 1 filter paper ($1\frac{1}{2}$″ width) which had previously been stretched on a horizontal plastic rack. Electrophoresis was carried out overnight in the cold at a current of 2.5 ma per strip. The buffer contained sodium diethylbarbiturate, sodium acetate, and sodium chloride in molal ratio of 2:1:1, and the pH was adjusted to 8.6 with 1N HCl. This buffer was selected on the basis of the observation that the presence of sodium chloride promoted clean separation of the albumin and α1-globulin components of rat serum. Optimal separation of components was attained at an ionic strength of 0.08, higher concentrations compressing the pattern and lower ones causing spreading of individual bands. Several types of apparatus, both commercial and homemade, were employed during the preliminary phases of the investigation. These included the techniques of tentlike suspension of the paper over a glass ridgepole, sandwiching of the paper between glass plates, and open horizontal suspension of the paper strips. The apparatus ultimately designed and used utilized the principle of open horizontal suspension, and yielded the most satisfactory results with tissue extracts. After electrophoresis, the strips were oven-dried (90° C) for ½ hour and stained with bromphenol blue.

A considerable diffusion artifact was introduced into the system by applying the test samples at the cathode end of the paper before buffer equilibrium had been reached. As the buffer flowed along the paper by capillary action the test sample was carried along with it, resulting in a generalized dislocation of the entire pattern relative to the origin. This compromise was resorted to because the patterns obtained by applying the sample to the fully equilibrated paper were far too diffuse. The method of Kunkel and Tiselius (13), in which the paper is sandwiched between glass plates, was found to be inadequate for use with tissue extracts because of the formation of tongues of proteinaceous material with little resolution of individual components. Reasonably good serum patterns were obtained regardless of the method.

Incubation of adenohypophyseal tissue with lysine-2-C^{14}

Groups of 8 adenohypophyses from normal and thyroidectomized rats were incubated for 3 hours at 37° C in Warburg flasks containing 0.1 μc. of lysine-2-C^{14} dissolved in 0.5 cc. of rat serum or saline. Following incubation, the tissue was washed in several changes of cold Ringer solution, and supernates were prepared and run electrophoretically as described above. The strips were oven-dried and, prior to staining, were

scanned with a Nuclear Actigraph and D-47 flow counter equipped with a micromil window. Activity was recorded through a rate meter connected to an Esterline-Angus ink writer. After scanning, the strips were stained and rescanned to make certain that the activity did not wash out of the paper. The radioactive peaks were then correlated with the stained components of the electrophoretic pattern. Samples of the medium were run electrophoretically and scanned in order to localize the position of the free amino acid and also to determine if any of the bound radioactivity might have been released from the tissue.

Hormonal activity of eluted electrophoretic components

Extracts of normal rat pituitary tissue were run electrophoretically and the paper strips, while still moist, were cut into sections corresponding to the components which had been located by staining duplicate strips. The sections were macerated in 2–3 cc. of acidified saline and kept in the refrigerator overnight. After centrifugation, the supernates were bioassayed for hormonal activity. Thyrotropin was estimated quantitatively by the effect of a single intravenous injection of the test sample on the uptake of I^{131} by the thyroid glands of male rats which had been pretreated with thyroid powder (14); and qualitatively by I^{131} uptake by the thyroid glands of hypophysectomized female rats.

Growth hormone was determined by the increase in width of the proximal tibial epiphysis in hypophysectomized female rats after 3 daily injections of the test sample. At autopsy, the tibias were split longitudinally in the mid-sagittal plane, fixed in Bouin's fluid, decalcified with 5% trichloroacetic acid and prepared for histological examination. The width of the epiphyseal disc was measured with an ocular micrometer. This is essentially the method of Kibrick *et al.* (15). Gonadotropic activity was detected by the increase in ovarian and uterine weights in hypophysectomized rats. For qualitative estimates of the localization of the pituitary hormones in the various electrophoretic components, it was convenient to carry out all the procedures in the same animals. No attempt was made to estimate ACTH, although adrenal weights were determined routinely in the assay rats.

RESULTS

Paper electrophoresis of pituitary extracts

Figure 1 illustrates the electrophoretic patterns of pituitary extracts of a variety of animals. Three major components were generally observed in the normal pattern. These studies will deal chiefly with the prominent slow band which appears consistently in all the patterns (indicated in Figure 1 by arrows). This band will be referred to below as the "thyroid-labile" component. Human and rat serum patterns are also included in Figure 1 to illustrate the dislocation of the entire pattern relative to the origin, and to show that the "thyroid-labile" component has a mobility equivalent to that of β-globulin.

Figure 2 demonstrates that the electrophoretic pattern of adenohypophyseal proteins is profoundly altered by the function status of the thyroid gland. Thyroidectomy or thiouracil-feeding leads to almost total disappearance of the "thyroid-labile" component, and thyroxine treatment returns it to its normal density. It was uniformly observed that the disappearance of the "thyroid-labile" component after thyroidectomy un-

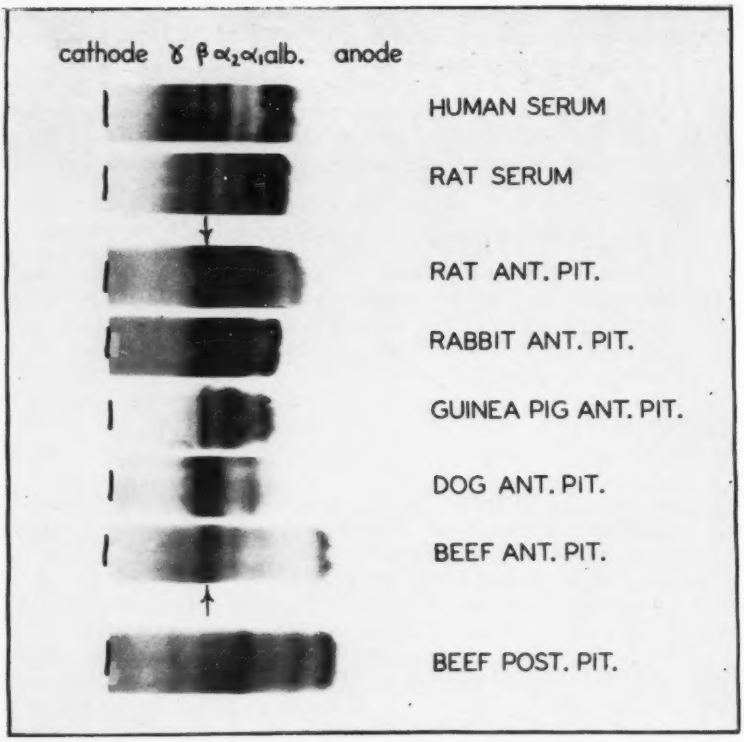

FIG. 1. Paper electrophoretic patterns of anterior pituitary extracts. Human and rat serum and beef posterior pituitary patterns shown for comparison. The origin is indicated by the vertical lines to the left of each pattern.

masked 2 or 3 faintly-staining bands which would be obscured in the normal electrophoretic pattern. Castration and adrenalectomy were without effect on the electrophoretic pattern.

The obviously poor resolving power of the paper electrophoretic technique reduces its value in the present studies. However, preliminary results have been obtained with starch gel electrophoresis run according to the method of Smithies (16). Figure 3 shows the excellent resolution of the protein constituents of rat pituitary extract (at least 9 bands were visible although all do not show in the photograph), and the dramatic disappearance of the "thyroid-labile" component following thyroidectomy. The 3 major components seen in the patterns obtained by paper electrophoresis (Fig. 1) were each cut out of the paper and re-run by the starch gel method. They retained their relative positions in the starch gel and could be matched with the components in the patterns obtained with whole extract run directly by the starch gel method. These findings will be reported in a future communication.

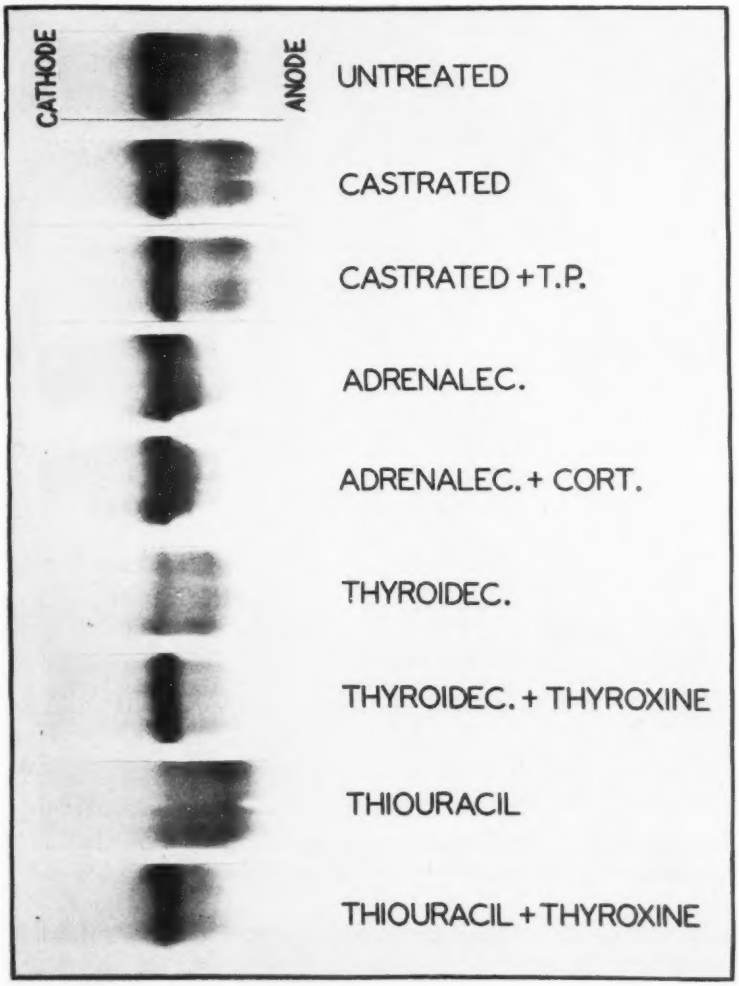

FIG. 2. Paper electrophoretic patterns of rat anterior pituitary extracts showing disappearance of "thyroid-labile" component after thyroidectomy or thiouracil feeding.

In vitro incorporation of C^{14}-labeled amino acids into pituitary proteins

Preliminary studies were carried out with isotopic alanine and phenylalanine. After 3 hours of incubation of pituitary tissue in serum containing either of these amino acids, there appeared to be some slight incorporation of radioactivity in the region of the "thyroid-labile" component of the pituitary electrophoretic pattern. However, the free amino acids also were localized in the same area. This meant that the radioactivity in the "thyroid-labile" component could have resulted from contamination, even

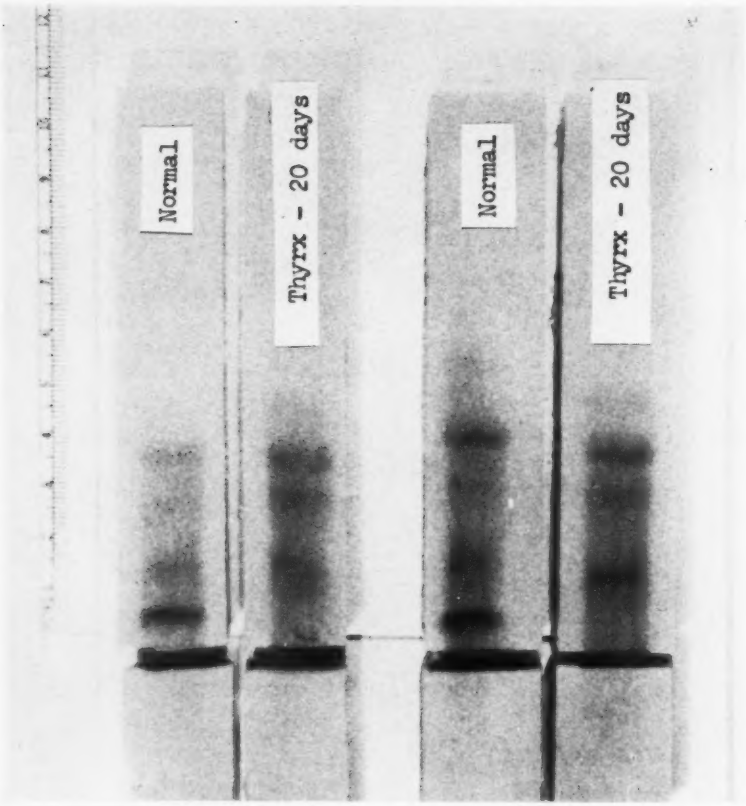

FIG. 3. Starch gel electrophoresis of rat anterior pituitary extracts. Patterns are shown for two different sets of normal and thyroidectomized rats.

though it could not be entirely washed out of the paper. Microdialysis techniques were investigated as a means of removing the unincorporated isotopic amino acids from the pituitary extract prior to electrophoresis, but the minute amount of extract available for each run (usually about 0.05 cc.) did not favor such an approach. This problem was circumvented by using another amino acid which could be handled more conveniently. Lysine was selected because, as a basic compound, it would migrate to the cathode at the pH employed for electrophoresis, while the proteins migrated to the anode. Thus, any radioactive peaks detected in the protein-staining area would represent protein-bound lysine.

Pituitary glands from normal rats were incubated for 15, 90, and 180 minutes in serum containing C^{14}-labeled lysine as described in the MATERIALS AND METHODS section above. Duplicate aliquots of the extracts were run electrophoretically for 3 or 16 hours. The 3-hour runs were stained

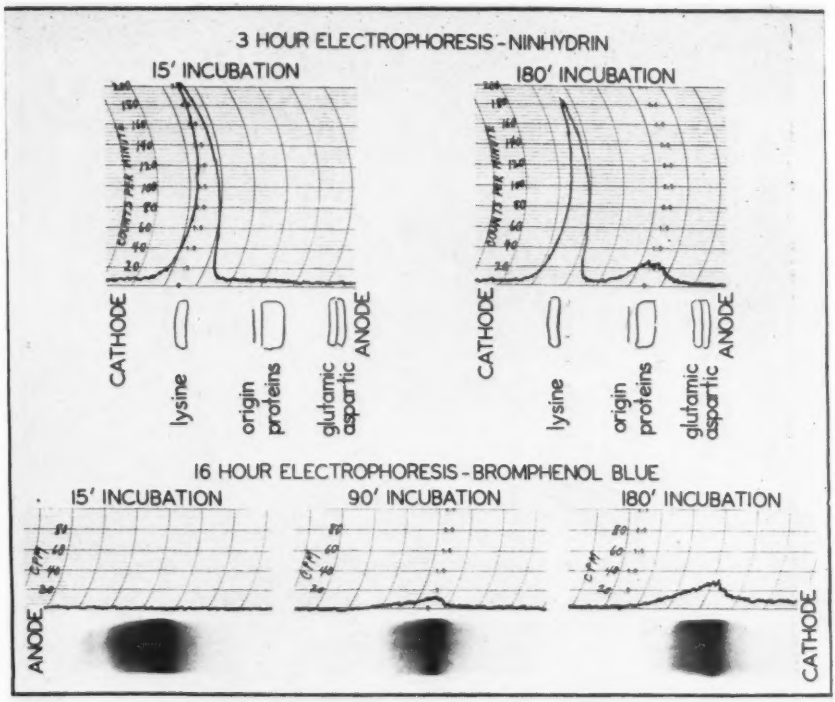

FIG. 4. Incorporation of lysine-2-C^{14} into soluble proteins of the rat anterior pituitary gland.

with ninhydrin to locate the position of free lysine and the 16-hour runs were stained with bromphenol blue. The ninhydrin-stained areas were marked artificially because of their tendency to fade. The upper tracings in Figure 4 show the large peaks of lysine radioactivity moving toward the cathode. A smaller peak persisted in the protein region in the sample incubated for 180 minutes. The lower tracings show that the protein-bound radioactivity increased with time of incubation, suggesting that the phenomenon was mediated by a biological (enzymatic) rather than a physical (adsorptive) process. The free lysine contaminant is not present in the lower tracings because electrophoresis was carried out for 16 hours and in that time the lysine migrated into the cathodal buffer vessel.

Figure 5 demonstrates that lysine incorporation occurs only if incubation is carried out in rat serum; no incorporation takes place if the tissues are incubated in Krebs-Ringer phosphate solution, even though respiration is equivalent in both media. In this experiment the strips were counted with a gas flow counter mounted on a support which automatically advanced the strips 0.5 cm. after each counting period.

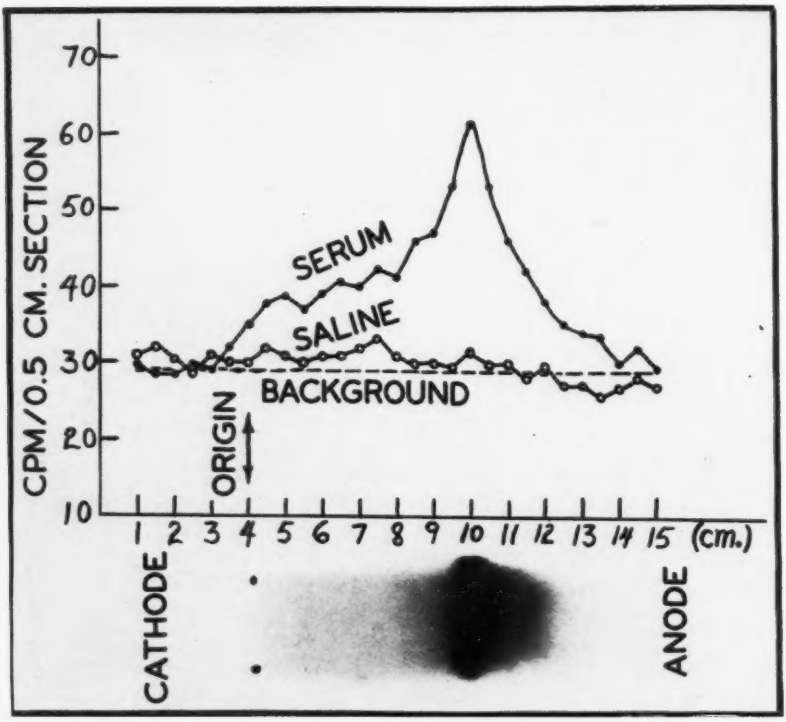

FIG. 5. Incorporation of lysine-2-C^{14} into pituitary proteins. Comparison of incubations in serum and saline.

In Figure 4 it is seen that the radioactive peaks after 90 and 180 minutes of incubation correspond to the center of the "thyroid-labile" component. This correspondence is also seen in Figure 5. It is difficult to ascertain from these figures whether there is specific incorporation of isotope into the "thyroid-labile" component or if the concentration of radioactivity is merely proportional to the staining intensity of the proteins. However, Figure 6 indicates that there is little difference in the incorporation of lysine into the area of the "thyroid-labile" component of normal and thyroidectomized rat pituitary extracts, even though there is a great difference in the amount of protein in this area in the two groups.

Hormonal activity of eluted electrophoretic components

A preliminary study was conducted on the quantitative recovery of thyrotropic activity from the eluted fractions of rat pituitary extract. The original extract was found to contain 200 U.S.P. milliunits of thyrotropic activity/0.02 cc. (as determined by bioassay). Duplicate samples of 0.02 cc of extract were run electrophoretically. One was stained with brompheno

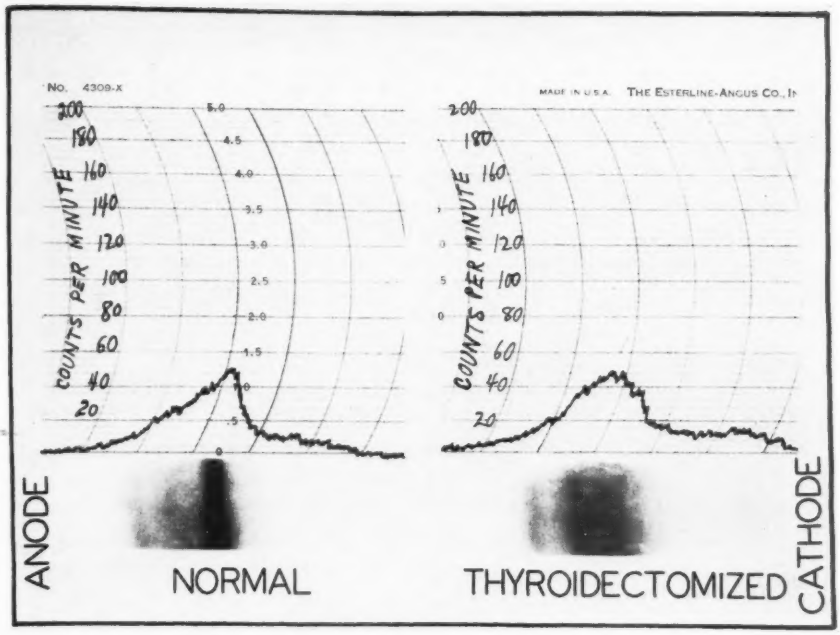

FIG. 6. Incorporation of lysine-2-C^{14} into pituitary proteins of normal and thyroidectomized rats.

blue to locate the protein components and the other was cut into sections corresponding to the main constituents on the stained strip. Figure 7 shows the stained strip, the division of the electrophoretic pattern into sections, and the recovery of thyrotropic activity from each section of the eluted strip. Most of the activity was in section 2, corresponding to the "thyroid-labile" component. The small amount of activity in section 1 probably represents trailing of the hormonally active material. Complete recovery of thyrotropic activity was obtained.

Table 1 summarizes the experiments in which simultaneous determination of thyrotropic, gonadotropic, and growth hormone activities were carried out in the same hypophysectomized rat. Groups V and VI show the results obtained with the growth hormone standard (Armour's Somar). The 0.2 mg. growth hormone standard was contaminated with about 75 U.S.P. milliunits of thyrotropin, as determined by thyroidal uptake of I^{131} (compare with Group III). Groups VII–IX illustrate the recovery of growth hormone and FSH (Armour Pituitary Gonadotropin—lot no. R377200) from the paper strip following electrophoresis. Duplicate samples of a mixture of 1 mg. of growth hormone and 3 mg. of FSH were subjected to electrophoresis. One strip was stained with bromphenol blue and the other was eluted in two sections, one corresponding to section 2 and the

TABLE 1. SIMULTANEOUS BIOASSAYS OF GONADOTROPIN, THYROTROPIN, AND GROWTH HORMONE IN HYPOPHYSECTOMIZED RATS

Group No.	Treatment	No. rats		B.W. (grams)	Mg./100 gm. body weight				Thyroid I^{131} uptake (% admin. dose per mg. thyroid)	Epiphyseal width (micra)
					Adrenal	Ovary	Uterus	Thyroid		
I	Intact control	13	Mean S.E.	158 ±28	27.5 ±1.9	35.9 ±2.4	138 ±37	5.39 ±1.25	0.233 ±0.093	419 ±68
II	Hypox control (15 days post-op.)	13	Mean S.E.	79 ±12	13.2 ±2.9	7.63 ±1.29	19.3 ±3.4	4.85 ±0.71	0.029 ±0.009	219 ±28
III	75 mu. Thyrotropin	3	Mean Range	83 75–91	17.9 16.9–19.4	9.59 8.40–10.71	23.4 22.3–24.9	5.98 5.07–6.48	0.096 .081–.108	211 202–214
IV	1200 mu. Thyrotropin	2	Mean Individual values	71 66, 75	15.1 16.4, 13.7	10.22 11.50, 8.93	19.9 21.4, 18.3	6.37 6.07, 6.66	0.359 .408, .309	211 226, 196
V	100 μg. Growth Hormone	5	Mean S.E.	92 ±2	13.2 ±1.5	6.40 ±0.48	21.6 ±1.1	4.62 ±0.73	0.065 ±0.010	311 ±20
VI	200 μg. Growth Hormone	5	Mean S.E.	92 ±2	12.3 ±0.8	6.77 ±1.28	23.2 ±3.7	4.87 ±0.66	0.093 ±0.017	359 ±44
VII	200 μg. Growth Hormone +600 μg. Gonadotropin-non-electrophoresed	5	Mean S.E.	93 ±5	11.1 ±1.3	20.6 ±5.4	82.2 ±11.1	5.52 ±0.20	0.148 ±0.049	389 ±14
VIII	200 μg. Growth Hormone +600 μg. Gonadotropin-electrophoresed, Section 2	4	Mean S.E.	94 ±5	13.8 ±2.2	16.9 ±1.8	90.3 ±6.3	5.03 ±0.46	0.136 ±0.050	378 ±27
IX	200 μg. Growth Hormone +600 μg. Gonadotropin-electrophoresed, Combined sections 3 and 4	4	Mean S.E.	80 ±3	12.2 ±0.9	6.29 ±0.68	19.7 ±0.9	5.06 ±0.36	0.039 ±0.023	212 ±8
X	Rat Pituitary Homogenate diluted in saline (6.12 mg. eq./rat)*	4	Mean S.E.	91 ±9	11.7 ±4.4	33.0 ±6.7	94.5 ±7.3	4.57 ±0.98	0.014 ±0.015	313 ±13
XI	Rat Pituitary Homogenate diluted in buffer (6.12 mg. eq./rat)	3	Mean Range	82 76–90	21.8 20.6–22.9	38.1 34.0–42.8	108 93–120	7.05 6.75–7.63	0.157 .134–.195	344 333–352
XII	Rat Pituitary Extract #1 (3.3 mg. eq./rat)	5	Mean S.E.	69 ±3	12.9 ±2.6	9.39 ±0.56	20.9 ±2.5	5.19 ±1.11	0.144 ±0.057	402 ±24
XIII	Rat Pituitary Extract #2 (5.5 mg. eq./rat)	3	Mean Range	95 93–97	11.5 11.4–11.8	7.36 6.88–7.98	21.3 20.0–23.2	5.74 5.22–6.29	0.187 .136–.215	351 342–360
XIV	Rat Pituitary Extract #2—electrophoresed, Section 2 (4.9 mg. eq./rat)	4	Mean S.E.	86 ±5	11.0 ±1.9	5.97 ±0.55	18.6 ±2.8	5.87 ±0.65	0.096 ±0.018	269 ±16
XV	Rat Pituitary Extract #2—electrophoresed, Combined sections 3 and 4 (4.9 mg. eq./rat)	4	Mean S.E.	74 ±4	12.0 ±0.8	6.12 ±0.97	20.2 ±3.7	5.52 ±0.96	0.027 ±0.008	218 ±9

* mg. eq. = the weight of fresh pituitary tissue equivalent to the volume of homogenate or extract injected.

other to the combined sections 3 and 4 in Figure 7. Each assay rat received $\frac{1}{5}$ of the total eluate in three divided daily doses. To check completeness of recovery a non-electrophoresed mixture of growth hormone and FSH was assayed along with the eluted fractions. Each rat in this group received 0.2 mg. of growth hormone and 0.6 mg. of FSH, representing $\frac{1}{5}$ of the amount of these substances applied to the paper for electrophoresis. Group VII shows the bioassay values for the non-electrophoresed mixture. Group VIII demonstrates that both growth hormone and FSH were located in section 2 and that recovery was virtually complete (compare with Group VII). Group IX indicates that no hormonal activity was recovered from combined sections 3 and 4.

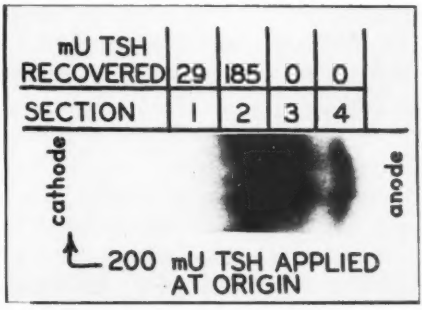

FIG. 7. Recovery of thyrotropic hormone activity from eluted components of the rat pituitary electrophoretic pattern.

The results of a series of experiments on rat pituitary homogenate and extract are seen in Groups X–XV. Gonadotropin, thyrotropin, and growth hormone were detected in the homogenate (Groups X and XI). All 3 of these activities were enhanced when the homogenate was diluted with the electrophoretic buffer as compared with saline. This was done to make certain that the buffer would not inactivate the hormones in subsequent electrophoretic studies. Gonadotropic activity was lost when the homogenate was centrifuged and only the supernate injected (Groups XII and XIII). However, thyrotropic and growth hormone activity were retained. Assay of the eluted components following electrophoresis of the supernate showed that thyrotropin and growth hormone were localized in section 2 (the "thyroid-labile" component), with no activity detectable in the combined sections 3 and 4 (compare Groups XIV and XV).

DISCUSSION

The technique of paper electrophoresis has been employed for the study of extracts of adenohypophyseal tissue. Satisfactory patterns, showing three major components in rat pituitary extracts, were obtained with a method involving free horizontal suspension of the paper strips. The slowest-moving of these constituents was referred to as the "thyroid-labile" component because it was reduced in concentration after thyroidectomy or thiouracil-feeding and was restored by thyroxine treatment. Castration and adrenalectomy were without effect in this regard.

The disappearance of the "thyroid-labile" component after thyroidectomy has not been fully explored but a tentative explanation can be ad-

vanced through consideration of several possibilities.

(1) The "thyroid-labile" component might be growth hormone, which was eluted from this region and which is known to decline in the absence of thyroid activity. However, this is unlikely because the decline in growth hormone content of rat adenohypophyses after thyroidectomy is not manifested for several months (17), whereas the "thyroid-labile" component disappears completely in 20 days.

(2) The "thyroid-labile" component might be thyrotropin, also recoverable from this region and which, in the rat, is sharply reduced after thyroidectomy as the result of its rapid discharge into the blood (12). This conclusion is also unwarranted because, while the thyrotropin content of the pituitary gland declines 50–75% after thyroidectomy, the "thyroid-labile" component disappears completely at an equivalent time period. In addition the small amount of thyrotropin in the rat pituitary gland in terms of weight could not account for the heavy concentration of the "thyroid-labile" component in the electrophoretic patterns. Our studies indicate that the adenohypophysis of the normal rat contains an average of about 200 U.S.P. milliunits of thyrotropin (with a range of 100–300 milliunits). This biological activity is equivalent to 200 μg. of Armour Thytropar or 135 μg. of the thyrotropin preparation used in making up the U.S.P. Thyrotropin Reference Standard. A 20-fold purification of thyrotropin would bring this value down to less than 10 μg. per pituitary gland. This degree of purification is not unlikely in view of investigations currently pursuing that objective (18, 19).

(3) The "thyroid-labile" component represents material identical with, or derived from, acidophilic granules (not necessarily growth hormone), which disappear completely 21 days after thyroidectomy (20). The parallelism in the disappearance of the acidophils and the "thyroid-labile" component after thyroidectomy suggests that these two phenomena might be directly related. Indeed, this might be an instance of the same protein constituent visualized by two different means, either intracellular staining or staining on filter paper following electrophoretic resolution.

The amino acid uptake study was conducted in order to determine if this could be related to the experimentally-produced fluctuations in the "thyroid-labile" component. However, the incorporation of lysine-2-C^{14} into the proteins in the region of the "thyroid-labile" component was the same in normal and thyroidectomized rats. It might be concluded, therefore, that the "thyroid-labile" component was not the one containing the isotope, but that the other constituents which normally are masked by the "thyroid-labile" component were the ones which incorporated the amino acid.

An interesting side feature of the isotope studies was the observation that lysine incorporation occurred when incubation was carried out in serum but not in saline buffer. Borsook and coworkers (21) have shown

that incorporation of several amino acids (including lysine) into the proteins of rabbit reticulocytes was enhanced in plasma as compared with saline. It was further demonstrated (22) that boiled liver filtrate stimulated amino acid incorporation into reticulocyte proteins. Fructose-amino acids were implicated as the stimulating factor in the liver filtrate but not in the plasma.

Our recovery of rat thyrotropin from an electrophoretic component with a mobility similar to the β-globulins of human or rat serum confirms the work of Lameyer *et al.* (23), who reported that human thyrotropic activity migrated with the β-globulin of human serum.

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PITUITARY FUNCTION IN THE THYROIDECTOMIZED RAT

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ABSTRACT

Male rats were thyroidectomized, gonadectomized or subjected to the combined operations of thyroidectomy and gonadectomy, and the productions and release of pituitary hormones was judged by the status of the endocrine organs, by the morphology of the pituitary, and by bioassay of trophic hormones in the pituitary and plasma.

1. After thyroidectomy (a) the pituitary target organs atrophied, (b) the plasma showed increased amounts of thyroid stimulating hormone, and decreased amounts of growth promoting substances, and (c) the anterior pituitary contained decreased thyrotrophic, follicle stimulating, interstitial cell stimulating and growth hormone content.
2. After gonadectomy (a) the accessory reproductive organs atrophied, (b) the plasma contained increased amounts of gonadotrophic, and normal amount of growth promoting substances, (c) the anterior pituitary contained increased follicle stimulating and interstitial cell stimulating hormone content and unchanged thyrotrophic and growth hormone content.
3. After thyroidectomy and gonadectomy (a) the endocrine and reproductive organs atrophied, (b) the plasma contained increased amounts of thyroid stimulating and gonadotrophic substances and decreased amounts of growth promoting substance, (c) the anterior pituitary contained increased amounts of follicle stimulating and interstitial cell stimulating hormones, and decreased amounts of thyrotrophic and growth hormones.
4. Histologically detectable evidence of the activity of the pituitary chromophils correlated well with the biological findings.

THE retardation in body growth of the thyroidectomized rat has been attributed to pituitary hypofunction. Dwarfism results from thyroidectomy and growth is reinstated by growth hormone. Thyroxin not only induces growth in the thyroidectomized rat but repairs the histological abnormalities of the anterior pituitary (1, 2, 3). The impaired development of the reproductive organs and adrenals has been likewise attributed to pituitary malfunction (1, 4, 5, 6). It has been assumed that due to the

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lack of the thyroid hormone the pituitrary does not release, possibly does not secrete, the gonadotrophic, adrenocorticotrophic and growth hormones, with resultant atrophy of the target organs and retardation in growth (7, 8). Direct evidence has not been presented to show whether the defect is due to reduced ability of the pituitary to produce and release hormones or of the target organs to respond to pituitary hormones in the absence of thyroxin.

It was the purpose of this study to determine the production and release of pituitary hormones in the thyroidectomized rat, as judged by the status of the target organs, the morphology of the pituitary, and by bioassay of trophic hormones in the pituitary and in blood plasma. Using these criteria an attempt was made to determine whether the pituitary of the thyroidectomized rat can respond after castration like that of the normal rat.

MATERIALS AND METHODS

Male rats of the Long-Evans strain maintained on stock diets of natural foodstuffs[2] were subjected to thyroidectomy, castration or to both operations. Their pituitaries were assayed for hormone content, and the blood plasma was simultaneously assayed for evidence of circulating hormone. All assays were conducted in hypophysectomized immature female rats.[3] The animals were 26–28 days old at operation. Injections were begun two weeks post-operatively and were given daily intraperitoneally for four consecutive days.

Operative procedures. Male rats were surgically thyroidectomized at 45 days of age, the operation involving the removal of the parathyroids as well as the thyroid. In all surgical procedures, one fourth of the donor rats were subjected to the operation on each of 4 consecutive days in order to have donors available after the same post-operative periods for the 4 day assay period. Bioassays were made of hormone content of the blood 21 and 56 days after thyroidectomy, when the rats were 66 and 101 days of age. Eighteen to 24 hours before autopsy the donors were injected with 1 μc. of I^{131}. At the time of autopsy tissues of the ventral neck region were placed in counting vials and the radioactivity of the region counted in the scintillation counter (9). The radioactivity was compared with that of a comparable mass of muscle from the leg and any positive difference was assumed to represent radioactivity taken up by thyroid remnants. Any rats showing radioactivity in the neck region of more than 0.05% of the normal I^{131} uptake, or 0.005% of the injected dose, were considered incompletely thyroidectomized and were

[2] All thyroidectomized, castrated or thyroidectomized-castrated donor rats were maintained on a modified McCollum Diet I, consisting of ground whole wheat 67.5%, casein 15%, skim milk powder 7.5%, sodium chloride iodized 0.75%, calcium carbonate 1.5%, melted fat 6.75%, fish oil (Vit. A and D concentrate) 1%. KI solution added to 1 μg. of iodine per gram of diet (450 mg. KI per liter, 300 cm.^{3} per 270.5 lbs. per diet). All rats received a supplement of lettuce once a week.

[3] Hypophysectomized rats used in assays were maintained on the following diet: ground whole wheat 68.5%, casein 5%, alfalfa leaf meal 10.0%, fish oil 5%, sodium chloride 1.5%. KI solution added to 1 μg. of iodide per gram of diet (450 mg. KI per liter, 300 cm.^{3} per 270.5 lbs. of diet). In addition a dish of wet mash of the previously described McCollum Diet I was offered every afternoon. They received a supplement of lettuce 3 times weekly. They were maintained at $74° \pm 1°$ F temperature.

not included in the donor group. Male rats were *gonadectomized* at 45 days of age and were sacrificed 21 and 56 days later when 66 and 101 days of age. In the groups subjected to *combined thyroidectomy and castration* the male rats were thyroidectomized at 45 days of age; thirty-five days later they were gonadectomized and were sacrificed 21 days later. This period after castration was found to be sufficient to increase the production and release of gonadotrophic hormones after gonadectomy of normal rats. *Normal* rats of the same age were maintained as controls and were sacrificed at the same age.

Collection of blood. Groups of donor rats were anesthetized on 4 consecutive days and blood was withdrawn from the aorta into heparinized syringes and immediately transferred into individual centrifuge tubes under cover of mineral oil. The blood was centrifuged for 10 minutes at 2500 rpm, the plasma was aspirated into syringes and immediately injected into the hypophysectomized assay rats; the time consumed between withdrawal and injection was less than 30 minutes.

At autopsy of the donors, testes, seminal vesicles, adrenals, thyroids and spleens were dissected and weighed.

The pituitaries were collected from all the donors immediately after the blood was withdrawn, not more than 3–5 minutes being allowed to elapse. The anterior pituitaries, except those saved for histological analysis, were dissected free of the posterior lobe, were frozen immediately over solid carbon dioxide and kept frozen until the time of bioassay. When groups of pituitaries were needed for bioassay they were weighed, and homogenized in saline. The volume injected was adjusted so that a total dose of 1/64, 1/32, 1/16, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$ or 1 anterior pituitaries was administered in a four day period.

Sixteen hours before the autopsy the hypophysectomized test animals were injected intraperitoneally with 0.5 μc. of I^{131}. The thyroids were dissected and placed in counting vials containing Bouin's fluid for counting in the scintillation counter (9). The results are presented as per cent uptake of the injected dose. The thyroids were later sectioned and stained with hematoxylin and eosin for histological examination. Ovaries and uterus were weighed, and ovaries were fixed in Bouin's fluid, sectioned and stained with hematoxylin and eosin for histological examination. Tibias were removed and stained with silver nitrate, and the width of the uncalcified portion of the proximal epiphyseal cartilage of the tibia was measured (10).

In the assays of plasma known amounts were given intraperitoneally daily for four consecutive days and the recipients were sacrificed 24 hours after the last injection. Eighteen hours before autopsy all animals were injected with 0.5 μc. of I^{131}. The thyroids, ovaries, uteri and tibias were examined by the same methods as in assays of pituitaries.

The completeness of hypophysectomy of the assay animals was judged by examination of the sella turcica for presence of pituitary fragments. The atrophy of the pituitary target organs furnished confirmatory data. Data from rats incompletely hypophysectomized were discarded.

In an attempt to investigate histologically the functional changes in the anterior pituitaries after thyroidectomy, pituitaries were analyzed from rats in each of the four groups—normal controls, thyroidectomized, gonadectomized and thyroidectomized-gonadectomized rats. Pituitaries were fixed in Zenker-formol and after nitrocellulose embedding were sectioned at four micra in a horizontal plane. Representative sections from five levels of the gland were stained with a modified Mallory-Azan stain (11) and also by the periodic acid Schiff stain (PAS).

RESULTS

Target Organs of Donors

Table 1 presents the data indicative of the endocrine status of the donor rats. As anticipated, atrophy of all the pituitary target organs was ob-

TABLE 1. ORGAN WEIGHTS OF THYROIDECTOMIZED ($\overline{T}$), GONADECTOMIZED ($\overline{G}$), THYROIDECTOMIZED-GONADECTOMIZED ($\overline{TG}$) AND NORMAL (N) MALE RATS

Group	Age, da.	Period after operation, da	Body weight gain, mg.	No. of rats	Pituitary, mg.	Testes, mg.	Seminal vesicles, mg.	Adrenals, mg.	Thyroid		Spleen, mg.
									I^{131} uptake %	Wt., mg.	
$\overline{T}$	66	21	47 ± 3	8	—	2571 ± 35	500 ± 15	24 ± 1	0.005	—	—
	101	56	66 ± 3	18	8.4 ± .5	2061 ± 40	343 ± 12	21 ± 1	0.002	—	753 ± 15
$\overline{G}$	66	21	79 ± 4	31	—	—	—	37 ± 2	9.43 ± .29	24.5 ± 1.2	—
	101	56	168 ± 8	10	8.2 ± .4	—	52 ± 3	42 ± 2	8.50 ± .31	34.1 ± 1.1	846 ± 21
$\overline{TG}$	101	56 21	50 ± 3	20	9.6 ± .4	—	71 ± 4	24 ± 1	0.005	—	653 ± 30
N	66	—	60 ± 4	20	—	2650 ± 40	440 ± 16	37 ± 2	9.30 ± .32	24.1 ± 1.8	1050 ± 22
	101	—	164 ± 6	16	7.1 ± .3	3006 ± 52	870 ± 20	42 ± 2	10.18 ± .51	38.1 ± 2.1	1358 ± 27

served after thyroidectomy. The adrenals weighed only 24 mg. as compared with 37 mg. in normal rats of the same age. The combined operations of thyroidectomy and castration resulted in approximately the same degree of atrophy of the adrenals, whereas castration alone did not alter adrenal weight. The testes in the thyroidectomized rats weighed 2061 mg. 56 days after operations whereas in controls of the same age they averaged 3006 mg. The weight of the seminal vesicles was approximately half normal at

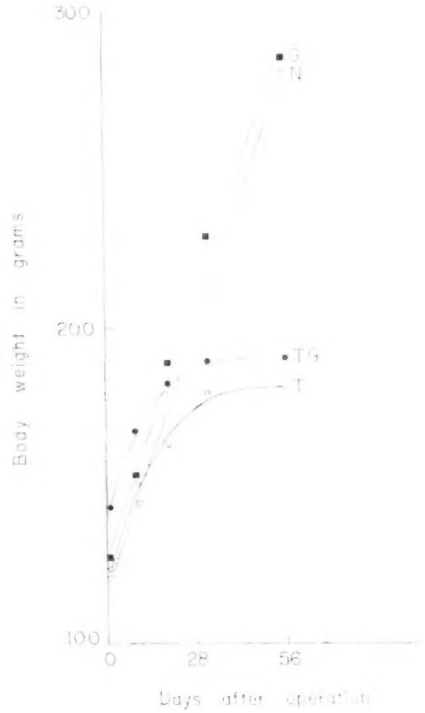


FIG. 1. Body growth of thyroidectomized ($\overline{T}$), castrated ($\overline{G}$), thyroidectomized-castrated ($\overline{TG}$) or normal (N) male rats.

the end of the experimental period, 343 mg. compared with the normal value of 870 mg. The seminal vesicles in gonadectomized rats were atrophic weighing 52 mg. and after combined thyroidectomy and castration the degree of atrophy of the seminal vesicles was similar to that after castration, 71 mg. The thyroids of castrated rats weighed the same as in normal rats (34.1 compared with 38.1 mg. in normals) and had the same I^{131} uptake (8.60 compared with 10.18%). As indicated in the described procedures, the percent of iodine uptake in thyroidectomized controls was less than 0.05% of the normal uptake. The pituitary weights were increased by all operative procedures. The normal increase in body weight was not affected by gonadectomy (Fig. 1). After thyroidectomy, or thyroidectomy and gonadectomy, there was a small body weight increase during the first 28 days, after which there was growth stasis. The spleens of thyroidectomized and of thyroidectomized and gonadectomized rats were decreased, to approximately half normal weight.

Plasma Assays

Table 2 shows in detail the evidence for trophic hormones (pituitary-like hormones) in the plasma of the different types of donors. The injection of

TABLE 2. PITUITARY-LIKE HORMONES IN THE PLASMA OF THYROIDECTOMIZED ($\overline{T}$), GONADECTOMIZED ($\overline{G}$), THYROIDECTOMIZED-GONADECTOMIZED ($\overline{TG}$) AND NORMAL (N) MALE RATS

Assayed in hypophysectomized rats, 4 day test

Donors			Hypophysectomized recipients						
					Ovaries*			Thyroid†	
Groups	Period after operation, da.	Daily plasma dose I.P. cc./da./4 da.	No. of rats	Tibial cartilage width, micra	Wt., mg.	Development	Uterus, mg.	I^{131} uptake %	Morphology
$\overline{T}$	21	2.7	4	165±5‡	8±1	sF	28±1	1.30±.11	+
	56	3.0	6	170±4	8±1	sF	27±1	3.25±.15	+
		4.0	7	178±4	9±5	sF	29±2	5.00±.18	+(++)
$\overline{G}$	21	1.0	4	170±6	8±1	sF	28±1	.41±.04	—
		3.0	4	200±6	12±2	s, mF	38±2	.30±.04	—
		6.0	3	210±3	28±2	m-1F	108±4	.92±.20	—
	56	3.0	5	195±4	17±1	s-mF	69±3	.30±.10	
		4.3	5	210±4	24±1.5	m-1F	86±5	.30±.05	—
		5.7	7	210±3	24±2	m-1F	90±4	.30±.06	
$\overline{T}$	56	1.8	5	170±5	11±1.8	s, sm, m	35±2	1.10±.10	+
		4.3	4	170±3	28±3.0	m-1F	104±6	4.00±.40	+(++)
$\overline{G}$	21	4.7	5	165±3	23±1.5	m-1F	111±3	3.40±.15	+(++)
N		1.0	6	172±2	8±0.5	sF	27±1	.44±.07	—
		3.2	5	204±3	9±0.5	sF	29±1	.55±.10	—
		4.5	6	207±3	9±1.0	sF	29±1	.45±.05	
		7.0	5	219±2	8±1.0	sF	30±1	.64±.08	
Uninjected Controls			15	150±5	8±1	sF	26±1	.50±.06	—

* Small, medium and large follicles are indicated by s, m, l, F; corpora lutea, CL; interstitial cells, deficient or repaired, IT def. or repair.

† Thyroid activity is designated one plus (+) when definite stimulation is present judged by the following criteria: the epithelium is low and the cytoplasm scanty; nuclei are slightly rounded and chromatin is less condensed than in hypophysectomized controls; the hematoxylin stain is somewhat paler, and the colloid stains a lighter eosin tint. The next grade of response is indicated by two plus (++); the epithelium is low cuboidal; nuclei are vesicular and light staining; vacuoles are beginning to appear in the colloid.

‡ S.E. $= \sqrt{\frac{d^2}{n(n-1)}}$

plasma from normal rats, even at the highest doses, 7 cc. daily, elicited no response in the thyroid gland of the recipient hypophysectomized rats, as judged by morphology or the I^{131} uptake. There was no increase in ovarian or uterine weight and no evidence of ovarian differentiation. The only positive finding was a significant increase in the width of the tibial epiphyseal plate after administration of 3.25 cc. of normal rat plasma (12).

The plasma from thyroidectomized rats contained increased amounts of thyrotrophic activity, and decreased amounts of growth promoting substance. The plasma drawn 21 days after operation given in 2.7 cc. daily doses for four days resulted in an I^{131} uptake of thyroids of recipient hypophysectomized rats of 1.30% of the injected I^{131} dose, an uptake significantly higher than that of non-injected controls (0.50%). Plasma from donors of longer post-operative periods caused a higher I^{131} uptake. When the thyroids of recipients of plasma were examined histologically they showed evidence of stimulation with an increase in the height of the cells and partial resorption of the colloid. Plasma from thyroidectomized donors caused no significant increase in the tibial epiphyseal widths of the recipients (12). (It should be noted, however, that at all levels there was a small increase in width of the tibial epiphysis of the recipient hypophysectomized rats.) The plasma of thyroidectomized rats caused no stimulation of the ovaries in the assay animals, judged by weight or morphology and the weights of the uteri remained the same as those of the uninjected controls.

The principal finding in assay of plasma of gonadectomized rats was an increase in follicle stimulating activity. The plasma when tested either at 21 or 56 days after operation caused an increase in ovarian and uterine weight when given in daily doses of 3 to 6 cc. Though large follicles were present at these doses there was no evidence of stimulation of interstitial tissue. As with normal plasma the doses injected produced no response in the thyroid gland as judged by morphology or I^{131} uptake. The tibial cartilage also responded to injection of plasma of gonadectomized rats to a degree similar to that observed from injection of normal plasma, i.e., a significant increase occurred when doses of 3 cc. or more were given daily.

Plasma from rats subjected to thyroidectomy and gonadectomy showed the combined trophic effects reported from injection of plasma from donors

TABLE 3. CONTENT OF PLASMA IN TROPHIC HORMONES IN THYROIDECTOMIZED, GONADECTOMIZED, AND THYROIDECTOMIZED-GONADECTOMIZED MALE RATS AS COMPARED WITH NORMAL VALUES

Group	Thyroid stimulating potency	Follicle stimulating potency	Growth promoting potency
$\overline{T}$	Increased	(none detected)	Decreased
$\overline{G}$	(none detected)	Increased	Unchanged
$\overline{TG}$	Increased	Increased	Decreased

subjected to the separate operations. The gonadotrophic potency of plasma was the same as after castration alone, the thyroid stimulating potency was of the same order as that after thyroidectomy, and the growth promoting potency was decreased as after thyroidectomy. The changes in trophic hormone content in plasma are summarized in Table 3.

Assays of Pituitaries for Hormone Content

The results of bioassays of anterior pituitaries of donors are shown in Table 4. The minimum amount of tissue from normal rat pituitaries necessary for thyroid stimulation in the assay rats was $\frac{1}{16}$ of an anterior

TABLE 4. TROPHIC HORMONES IN ANTERIOR PITUITARIES OF THYROIDECTOMIZED ($\overline{T}$), GONADECTOMIZED ($\overline{G}$), THYROIDECTOMIZED-GONADECTOMIZED ($\overline{TG}$), AND NORMAL MALE RATS (N)

Fraction of anterior pituitary	Type of rat	Thyroids†		Ovaries*		Uterus weight, mg.	Epiphyseal plate width, micra
		I^{131} uptake	Hist.	Weight, mg.	Hist.		
1	$\overline{T}$	4.07 ± .2	+(++)	20 ± 1	m-1F; IT repair	75 ± 3	190 ± 3
	$\overline{G}$	7.27 ± .3	++	80 ± 4	1F; CL	127 ± 6	240 ± 5
	$\overline{TG}$	5.18 ± .2	+(++)	62 ± 3	1F	133 ± 8	200 ± 2
	N	7.77 ± .2	++	34 ± 2	m-1F; CL	170 ± 10	262 ± 7
1/2	$\overline{T}$	2.60 ± .15	+	11 ± 1	sF; IT def.	31 ± 1	185 ± 3
	$\overline{G}$	5.38 ± .18	+	60 ± 3	1F; CL	122 ± 7	252 ± 7
	$\overline{TG}$	2.05 ± .17	+	18 ± 2	m-1F	73 ± 4	161 ± 3
	N	5.33 ± .3	+(++)	49 ± 3	1F; IT repair	160 ± 9	236 ± 5
1/4	$\overline{T}$	1.04 ± .06	−(±)	12 ± 1	sF; IT def.	29 ± 1	159 ± 5
	$\overline{G}$	2.28 ± .07	+	15 ± 2	s-mF; IT repair	46 ± 2	200 ± 4
	$\overline{TG}$	1.98 ± .09	−(+)	14 ± 1	s-mF; IT repair	29 ± 3	154 ± 6
	N	2.65 ± .15	+	12 ± 1	sF; IT def.	29 ± 3	228 ± 3
1/8	$\overline{T}$	0.83 ± .04	−	10 ± 1	sF; IT def.	26 ± 1	152 ± 4
	$\overline{G}$	1.30 ± .06	+	10 ± 1	sF; IT def.	29 ± 1	195 ± 5
	$\overline{TG}$	0.86 ± .04	−	10 ± 1	sF; IT def.	29 ± 1	149 ± 4
	N	2.30 ± .09	+	11 ± 1		29 ± 1	220 ± 3
1/16	$\overline{T}$	0.73 ± .04	−	−		−	148 ± 5
	$\overline{G}$	1.04 ± .08	+	−		−	192 ± 3
	$\overline{TG}$	1.62 ± .05	−	−		−	160 ± 5
	N	1.06 ± .09	±	−		−	182 ± 5
1/32	$\overline{T}$	0.47 ± .05	−	−		−	145 ± 1
	$\overline{G}$	0.54 ± .07	−	−		−	180 ± 4
	$\overline{TG}$	0.47 ± .03	−	−		−	153 ± 4
	N	0.39 ± .04	−	−		−	161 ± 6
0[1]	—	0.37 ± .05	−	9 ± 1	sF; IT def.	27 ± 1	155 ± 5
	—	0.37 ± .08	−	8 ± 1	sF; IT def.	27 ± 1	149 ± 3
	—	0.53 ± .07	−	10 ± 1	sF; IT def.	21 ± 1	155 ± 5
	—	0.36 ± .06	−	10 ± 1	sF; IT def.	23 ± 1	149 ± 3

[1] Controls were repeated with assays of each type of animal.
See footnotes to table 2.

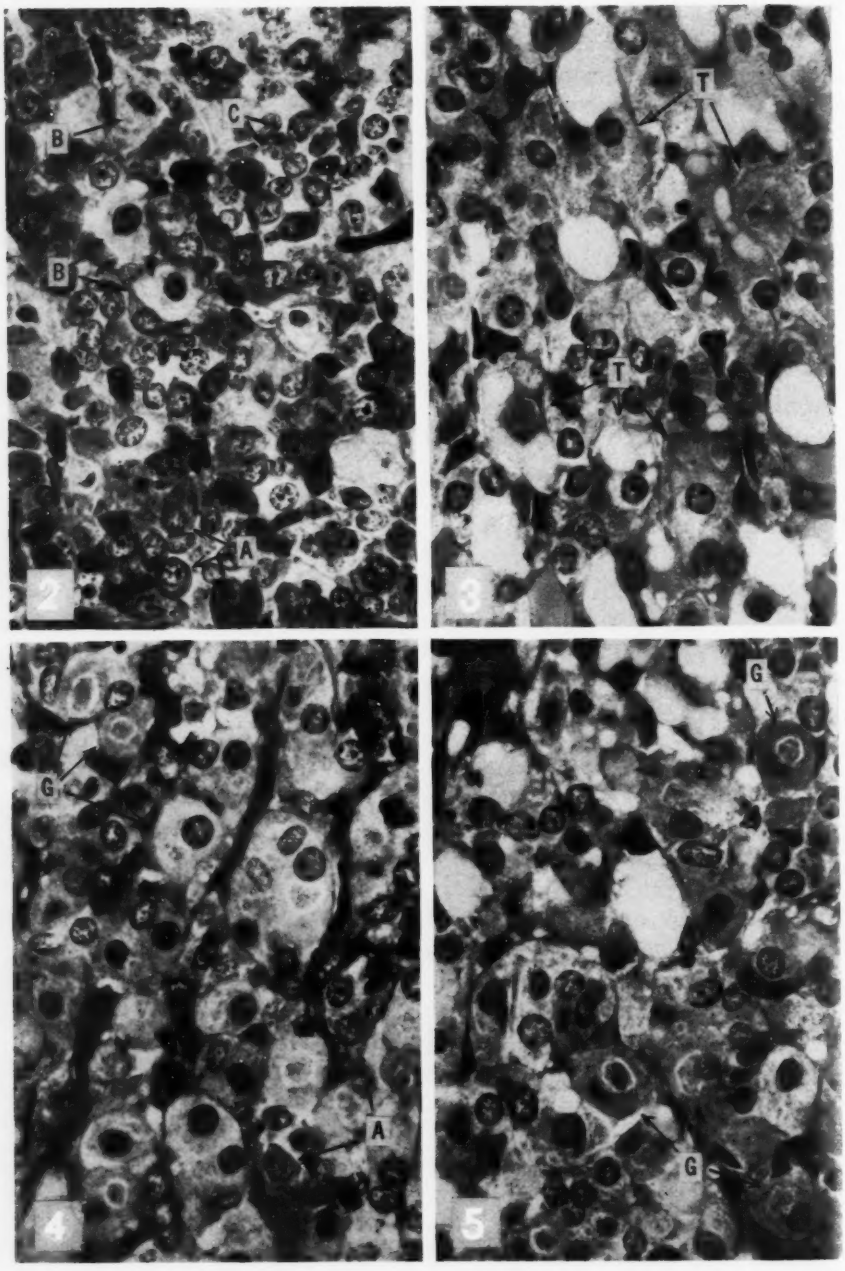
pituitary. This dose caused a significant increase in uptake of I^{131}. The thyroid epithelium was low cuboidal and cytoplasm was scanty, but the nuclei were slightly rounded and the chromatin was more dispersed than in thyroids of hypophysectomized controls. The nuclei stained somewhat paler with the hematoxylin and the colloid stained less intensely with eosin than in thyroids of controls. These findings have been designated in the tables by a single plus (+). The pituitaries of castrate rats elicited the same amount of thyrotrophic stimulation as normal pituitaries when judged by the same criteria. The thyrotrophic content of the pituitaries from thyroidectomized rats was decreased. A total dose of $\frac{1}{4}$ anterior pituitary gave minimal or no histological stimulation and minimal increase in the I^{131} uptake. The same decrease in thyrotrophic potency was found in the pituitaries of thyroidectomized-gonadectomized rats, the minimum effective dose necessary for thyroid stimulation judged both by morphological evidence and I^{131} uptake being greater than $\frac{1}{4}$ and less than $\frac{1}{2}$ donor pituitary glands.

The content of follicle stimulating hormone of the anterior pituitaries was judged by the minimum amount of tissue necessary to initiate the follicle growth in the ovaries of hypophysectomized immature rats (13). It was found that a total dose of $\frac{1}{2}$ anterior lobe ($>\frac{1}{4}<\frac{1}{2}$ gland) from normal male rats was necessary to initiate follicular growth. After thyroidectomy (56 days p.o.) 1 pituitary ($>\frac{1}{2}<1$ gland) was necessary to initiate follicular growth. When gonadectomy was superimposed on thyroidectomy the content of the pituitary in FSH was increased to the level found in the gonadectomized male rat, a total dose of $\frac{1}{4}$ anterior lobe being adequate to initiate follicular growth.

The interstitial cell stimulating potency (ICSH) of the pituitaries was judged by the dose necessary to initiate repair of the interstitial cells of the ovary (14). It was found that thyroidectomy resulted in a decrease in the ICSH content of the pituitary ($\frac{1}{2}$ normal rat gland was required for stimulation, 1 gland necessary after thyroidectomy). The content of ICSH in the pituitary after thyroidectomy-gonadectomy attained the same high level observed after castration alone; repair of interstitial cells was observed at the level of $\frac{1}{4}$ gland. The weights of uteri of recipients also indicated an increase in gonadotrophic content in thyroidectomized-gonadectomized comparable to that in gonadectomized rats' pituitaries.

The growth promoting potency of the pituitaries of normal rats was sufficient to increase the width of the proximal epiphyseal cartilage plate of the tibia at a total dose of $\frac{1}{16}$ to $\frac{1}{8}$ anterior pituitary. This minimal effective dosage was not changed after gonadectomy. Thyroidectomy alone, or combined with gonadectomy, resulted in a decrease in the amount of growth promoting potency, a total dose of about 1 gland being required to induce significant increase in width of the cartilage plate.

Table 5 summarizes the minimal effective doses of the trophic hormones



Photographs were taken with 4 mm. oil immersion objective; magnification ×600. Mallory-Azan stain.

TABLE 5. MINIMAL EFFECTIVE DOSES OF TROPHIC HORMONES IN THE ANTERIOR PITUITARIES OF THYROIDECTOMIZED (T), GONADECTOMIZED ($\overline{G}$), THYROIDECTOMIZED-GONADECTOMIZED ($\overline{TG}$) AND NORMAL (N) MALE RATS

Group	TSH	FSH	ICSH	GH
$\overline{T}$	1/4	>1/2<1	1	1
$\overline{G}$	1/16	1/4	1/4	>1/15<1/8
$\overline{TG}$	1/4	1/4	1/4	1
N	1/16	>1/4<1/2	1/2	>1/16<1/8

in the anterior pituitaries of rats subjected to the different experimental conditions. A fourfold decrease in thyrotrophic potency was noted after thyroidectomy, which is not influenced by a superimposed gonadectomy. The doubling of the gonadotrophic potency (both of FSH and of ICSH) following castration was not impaired by the thyroidectomy. The growth promoting potency of pituitaries after thyroidectomy was $\frac{1}{8}$ to $\frac{1}{16}$ normal and was not influenced by simultaneous removal of the gonads.

Histological Changes in Pituitaries

The main histological alterations found in the pituitaries of thyroidectomized and gonadectomized rats were similar to those described in the literature. In brief, after gonadectomy there was an increase in number and size of the basophils, and the PAS reaction was intense. Little if any change occurred in the acidophils (Figs. 2, 4). After thyroidectomy both chromophils were altered. The acidophils underwent extreme degranulation and became unrecognizable. The basophils increased in number, many were enlarged and the cytoplasm contained multiple hyalin vacuoles. An enlarged Golgi apparatus was not an uncommon finding in the vacuolated basophils (Fig. 3). The PAS stain was not intense. The basophils showing these changes were interpreted as thyrotrophs. The other basophils, which according to Purves and Griesbach (15, 16) on the basis of morphology and geographical distribution should be considered gonadotrophs, also

FIG. 2. Field from a section of anterior pituitary of a normal rat to show general distribution of cells. A—acidophils· B—basophils; C—chromophobes.

FIG. 3. Field from a section of anterior pituitary of a rat 7 weeks following thyroidectomy. Note complete absence of acidophils. Basophils are increased in number and size and contain multiple hyaline vacuoles prominent nucleoli and large Golgi apparatus.

FIG. 4. Field from a section of anterior pituitary of a rat 3 weeks following gonadectomy. Note considerable increase in number of basophils, and prominent negative image of the Golgi apparatus (G). Individual acidophils (A) appear unchanged but few are seen among the large basophils.

FIG. 5. Field from a section of anterior pituitary of a rat thyroidectomized 7 weeks previously and gonadectomized 3 weeks prior to autopsy. Basophils showing the typical effects of thyroidectomy can be seen in the upper left corner of the photograph. Numerous basophils (G) identical in appearance with "gonadectomy basophils" are present in the remainder of the field. No acidophils were found in glands of this group.

showed deviation from normal. They appeared to have decreased in number; the Golgi apparatus was small and the reaction with PAS was faint. Planimeter measurements of camera lucida tracings confirmed the decrease in size. These gonadotrophs in thyroidectomized rats averaged 248 planimeter units, compared with 335 units in normal control pituitaries, with no overlapping values from groups of 5 rats. When gonadectomy was superimposed on thyroidectomy, many of the basophils, supposedly gonadotrophs, underwent the same changes as in the castrated rats. Figure 5 of the plate clearly shows numerous enlarged basophils identical morphologically with the typical early castration cells seen in Figure 4. The other changes characteristic of thyroidectomy had not been altered; no acidophils were identifiable and many basophils showed multiple hyaline vacuolation.

DISCUSSION

The origin of the growth hormone is usually ascribed to the acidophils and their degranulation after thyroidectomy is associated with the cessation of growth. The lack of acidophils paralleled the decreased growth promoting potency of the pituitaries and plasma of thyroidectomized and thyroidectomized-gonadectomized rats. Since the potency of plasma was judged by the tibia test the absence of circulating thyroxine in the plasma might in part be responsible for the decreased potency of plasma in growth promoting substances, since thyroxin has a synergistic effect on the presence of growth hormone (17).

Though the gonadotrophic content of the pituitary of thyroidectomized rats was impaired as shown by bioassay, and pituitary gonadotrophic secretion was impaired as shown by the status of the target organs, both content and secretion were reinstated, even increased above normal, by superimposed gonadectomy. These observations are interpreted as showing that the anterior pituitary can produce and release gonadotrophins in the absence of thyroid hormone.

The histological evidence of decreased activity of gonadotrophic basophils after thyroidectomy paralleled the subnormal status of the gonads and the accessory reproductive organs. Both pituitary gonadotrophic hormone production and the histological evidence of activity of those basophils interpreted as "gonadotrophs" were reinstated when gonadectomy was superimposed on thyroidectomy. The typical thyroidectomy changes observed in many of the basophils (the "thyrotrophs") were not altered by superimposition of gonadectomy, and the thyrotrophic potency of the pituitary and the plasma was not different from that of thyroidectomized animals.

After thyroidectomy the content of the pituitary in thyrotrophic hormone was decreased whereas that of plasma was increased. On the other hand, after gonadectomy the content of gonadotrophins in both the pituitary and plasma was increased. If one can assume that the half life of the

trophic hormones is not changed after thyroidectomy or after gonadectomy, and that increased production and release of thyrotrophin and gonadotrophin occur after both thyroidectomy and gonadectomy, then the cells of origin of these hormones must differ in respect to storage; the increased content of gonadotrophin in castrated rat's pituitary coupled with increased content in plasma must represent storage as well as increased secretion by "gonadotrophs," whereas in the pituitary of the thyroidectomized rat the decreased pituitary potency coupled with increased thyrotrophic content in plasma must represent increased secretion without storage in the "thyrotrophs."

According to this interpretation the basophils which undergo extensive vacuolation after thyroidectomy are regarded as active, not degenerate cells. The nucleus is vesicular and the Golgi apparatus can frequently be seen to be enlarged. Griesbach and Purves from histological and physiological studies have considered them to be very active cells (18). The pituitary of the thyroidectomized rat has also been shown by study of parabiotic rats to be able to produce sufficient thyrotrophic hormone to maintain the thyroid of the hypophysectomized partner in a normal state (19).

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EFFECTS OF GROWTH HORMONE ON GLUCOSE UTILIZATION IN FASTED RATS[1]

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ABSTRACT

A single injection of growth hormone to fasted rats leads to a hypoglycemic response. Using a method of determination of rate of glucose utilization recently developed it can be shown that this hypoglycemic response is due to an increase in glucose utilization. Pretreatment of the animals with growth hormone decreases the response to a subsequent injection both as regards hypoglycemic reaction and as regards increase in glucose utilization.

A NUMBER of authors (1, 2, 3, 4) have observed hypoglycemia in fasted rats following administration of a single dose of growth hormone. Simultaneous growth hormone injection has also been shown to decrease the rise in blood sugar due to glucose injection (5). These findings are in apparent contradiction with the well known observation that chronic growth hormone administration can induce permanent diabetes in adult cats and dogs. Interpretation of the mechanism of this hypoglycemic effect is not available from *in vitro* experiments: in these, growth hormone had either been injected prior to sacrifice of the animals or was added in vitro; enhancement of glucose uptake, inhibition of glucose uptake and absence of modification of glucose uptake have been variously described as consequences of growth hormone administration or addition (6, 7). *A priori*, there are two alternative explanations for this hypoglycemia: increase in glucose utilization by the tissues or decrease in glucose output by the liver. It was the purpose of this study to test the first hypothesis *in vivo*. Availability of a method which expresses glucose utilization as a single number permits statistical processing of the data to be done more easily and more rigorously than can be done with the results of classical glucose tolerance curves. In this study, measurements of rate of glucose utilization were conducted after periods of various duration following the injection of

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growth hormone. The effect of pretreatment of the rats by growth hormone on the response to a subsequent injection was also examined.

Principle of the Method

The rate of disposition of glucose was determined by administering rapidly a relatively small intravenous glucose load (8, 9, 10). Glucose levels decrease exponentially with time. When this function is plotted on a semilogarithmic scale, it is transformed into a straight line. If G is the level of blood glucose at time t, then the function $G = G_0 e^{-Kt}$ becomes $\ln G = \ln G_0 - Kt$ and the velocity constant K can be determined from the levels G_1 and G_2 at times t_1 and t_2.

$$K = \frac{2.3(\log G_1 - \log G_2)}{t_2 - t_1}.$$

Actually, K can be determined with much greater precision if the least square method is used on a large number of points (in practice, of the order of 10), or if these points are used to determine the slope of the best fitting straight line graphically. To conform with the accepted expressions of concentration, K can be expressed as percentage of blood glucose decrease per minute. This rate has been considered an index of the amount of "effective" insulin bound by the tissues. The value of K rises when insulin is administered, and is decreased in diabetes. It is essential to note that provided the dose of glucose is relatively small, the glucose is given intravenously and the administration is relatively rapid, the value of K is reproducible, does not vary with the dose given, and does not vary when the test is repeated. By contrast, if glucose is given by mouth or in large, slowly administered intravenous doses, the subject presents the Straub-Traugott effect, a progressive decrease in the hyperglycemia following repeated administration of equal doses. Such an effect, if analyzed by the method used here would be translated into increases in the successive values of K(8).

MATERIAL AND PRODECURE

Wistar female plateaued rats (230–340 gm.) fed Purina chow were divided into five groups containing 9 to 11 animals. The rats were fasted for 21 hours prior to each glucose tolerance test. The taking of tail blood samples was conducted under light nembutal anesthesia. Immediately after the first tail sample had been obtained, a dose of 0.5 gm. glucose/Kg. body weight in 50% solution[3] was injected into the saphenous vein. Nine other 0.1 ml. samples were taken within the next 30 minutes and blood glucose was determined by the Somogyi-Nelson procedure (11, 12). In Group I these tests were performed immediately before and directly after the intravenous injection of growth hormone. In the remaining four groups the preinjection glucose tolerance test was performed a few days before and repeated on Group II 2 hours, groups III 5 hours, and Group IV 22 hours after a subcutaneous injection of the hormone. Another Group (V) received a subcutaneous injection of growth hormone for 3 days, then was tested under the same conditions as Group III (5 hours after the last administration). The bovine growth hormone used (NIH–BGH-1) was the highly purified material distributed by the Endocrinology Study Section, National Institutes of Health.[4] The powder was diluted in isotonic sodium chloride and kept at −20° C for up to 6 days. The amount injected was 1.5 mg. per animal in all cases.

[3] Solution of *d*-glucose (wt./vol.). Parke, Davis & Company, Detroit, Michigan.

[4] The authors gratefully acknowledge the gift of this material.

TABLE 1. ROLE OF THE TIME FACTOR ON THE EFFECTS OF GROWTH HORMONE (1.5 MG./ANIMAL) ON BLOOD SUGAR G AND GLUCOSE UTILIZATION K OF ADULT FEMALE RATS FASTED TO 22 HOURS

Group	*n*	Mode of administration	*t*	G_1	G_2	G_2-G_1	*p*	K_1	K_2	K_2-K_1	*p*
I	9	IV	0:10	59± 7	—	—	—	2.1±0.5	2.2±0.9	0.1±1.1	
II	9	SC	2:00	57± 9	45±17	12±17		2.1±0.5	2.7±0.7	0.6±0.8	<0.05
III	10	SC	5:00	66±10	45±10	20±20	<0.02	2.0±0.4	3.7±1.0	1.7±1.3	<0.01
IV	11	SC	22:00	65±10	50±14	15±14	<0.01	2.0±0.4	2.5±1.2	0.5±1.4	
V*	11	SC	5:00	57± 9	51± 8	6±10		2.2±0.6	2.9±0.6	0.7±0.9	<0.05

t=time interval between the injection of GH and the second glucose tolerance test.
G_1 and G_2: fasting blood sugars immediately before the first and second tolerance tests.
K_1 and K_2: rate of glucose utilization before and after GH, expressed as a percentage/100 ml. blood/minute (see text).
* The rats in this group have been injected with GH 5 hours previously, as in group III, but this injection is in this case the last of a series administered on each of 3 consecutive days (see text).

RESULTS

The type of data obtained in a representative animal is shown in Figure 1. General results are reported in Table 1. In agreement with results of other workers the fasting blood glucose (G) dropped 2 hours after growth hormone administration (Group II) and remained at similarly low levels after 5 and 22 hours (Groups III and IV). On the other hand, the group pretreated with growth hormone (V) showed less decrease in blood

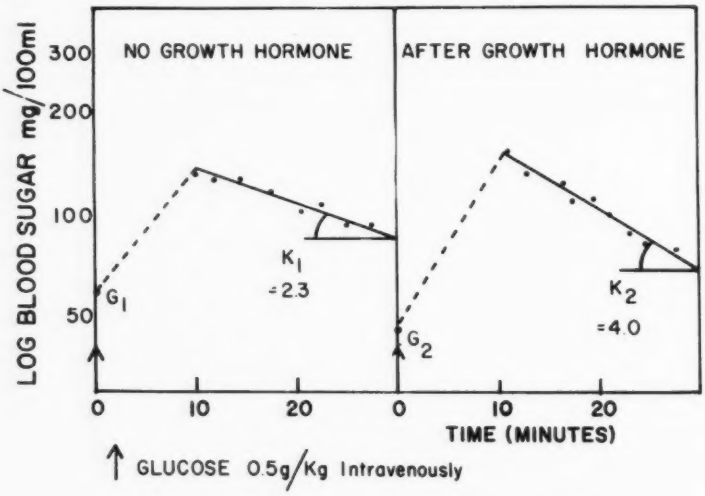

FIG. 1. Effect of a single injection of growth hormone on the rate of glucose utilization in a fasted adult female rat.

glucose under the same conditions. The rate of glucose utilization, K, was unmodified in the group of rats retested immediately after the hormonal treatment (Group I). There was a significant rise in rate of glucose utilization in the group tested 2 hours after growth hormone administration (Group II). The rise persisted in the group tested 5 hours after growth hormone injection (III); rates had declined to near normal values 22 hours after administration (IV). Pretreatment with growth hormone (Group V) decreased but did not eliminate the significance of the rise in utilization K.

DISCUSSION

The dose of growth hormone of approximately 5 mg./kg. used in this study appears high when one considers that the daily secretion of GH by young rats is estimated as 200 μg. (13). However, a daily dose of 1 mg. per plateaued female adult rat is one of the lowest being effectively measured by the bioassay based on the resulting weight increase (14). The results obtained with the 5 mg./kg. dose must therefore have some physiological significance.

The occurrence of a hypoglycemic response after a single dose of GH in fasted adult rats has been confirmed. This hypoglycemic response appeared in turn to be due to an increase in the rate of glucose utilization. These results can be compared to those obtained by Ross (15) in rabbits and Altszuler (16) in hypophysectomized dogs. Ross showed an increase in rate of transfer of glucose in the aqueous humor of the anterior chamber of the eye of two animals after 2 subcutaneous injections of anterior pituitary extracts. Altszuler showed an increase in C^{14}-labeled glucose uptake in dogs continuously perfused following a 4- or 5-day course of daily intramuscular growth hormone injection. The intimate mechanism of acute growth hormone effect on carbohydrate metabolism remains a matter of controversy. According to various authors, the hypoglycemia produced by growth hormone administration in rats seems dependent upon the presence of a normal pancreas (3) but is not necessarily mediated through an increase in insulin secretion (17, 18). In the present experiment, the effects of a single injection of growth hormone, although in the same direction as those that would be expected after an injection of a small dose of crystalline insulin—i.e. a lowering of the blood sugar and an increase in the rate of glucose utilization K (8)—required 2 hours to become manifest while insulin acts immediately. In contrast also with insulin, the repetition of injections of bovine growth hormone decreased the magnitude of the response.

A primary stimulating effect of growth hormone on the hepatic outflow of glucose, with a resulting stimulus for increased insulin secretion (7, 16) seems unlikely since the observed hypoglycemia indicates an inadequate hepatic homeostasis. Whatever the exact mechanism, it appears that if

there is an inhibitory effect of GH on the carbohydrate metabolism in many *in vitro* experiments, this is definitely masked by an opposite process in intact rats. Thus, the increase in the glycogen content of muscle *in vivo* reported by various authors (4, 19) under conditions similar to those in this experiment could be related to an enhanced glucose uptake, and not to a decreased carbohydrate utilization.

Finally, it is interesting to speculate on the possibility that the increase in rate of glucose utilization (K) previously reported in pregnant, fasted rats (20) may be related in part to the increase in blood growth hormone activity (21) observed in such animals.

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ASCORBIC ACID DEFICIENCY AND ADRENOCORTICAL FUNCTION IN THE GUINEA PIG[1]

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ABSTRACT

Aspects of ascorbic acid and 17-hydroxycorticosteroid metabolism have been studied during ascorbic acid deficiency in young, growing guinea pigs. Morphologic evidences of ascorbic acid deficiency were seen in the epiphyses before the onset of weight loss and before marked reduction occurred in ascorbic acid content of adrenal and liver. At this time, however, there was only a slight elevation in 17-OHCS of plasma and bile, and the elevation of plasma 17-OHCS following the administration of C^{14} labeled bacterial polysaccharide and of ACTH was of normal character. No evidence of alteration of cortisol-4-C^{14} metabolism was found in guinea pigs with early ascorbic acid deficiency.

IN THE guinea pig with advanced scurvy the adrenals enlarge (1, 2), the urinary excretion of 17-ketosteroids (3, 4) and formaldehydogenic substances (5) is increased and the plasma 17-hydroxycorticosteroids (17-OHCS) are elevated (6). The increase in urinary excretion of 17-hydroxycorticosteroids and formaldehydogenic steroids occurs late in the course of ascorbic acid deficiency at the time when the guinea pigs are losing weight. The plasma 17-OHCS have only been studied in late, acute scurvy, during the terminal period of rapid weight loss (6). Extremely high plasma levels of 17-OHCS have also been found in moribund patients (7). Ascorbic acid deficiency in its late stages, therefore, may simply act as a non-specific "stress" (8).

The present study concerns the effects of the early stages of ascorbic acid deficiency, prior to the onset of weight loss, upon a) the ascorbic acid content of the adrenal and liver, b) 17-OHCS levels of plasma and bile, c) the plasma levels of 17-OHCS following the administration of ACTH and of C^{14}-labeled *K. pneumoniae* polysaccharide, d) the plasma clearance and tissue distribution of the labeled bacterial polysaccharide.

MATERIAL AND METHODS

Guinea pigs of either sex maintained on a diet of Purina rabbit chow with daily supplements of cabbage were used as control animals. Animals, weighing 150–250 gm.

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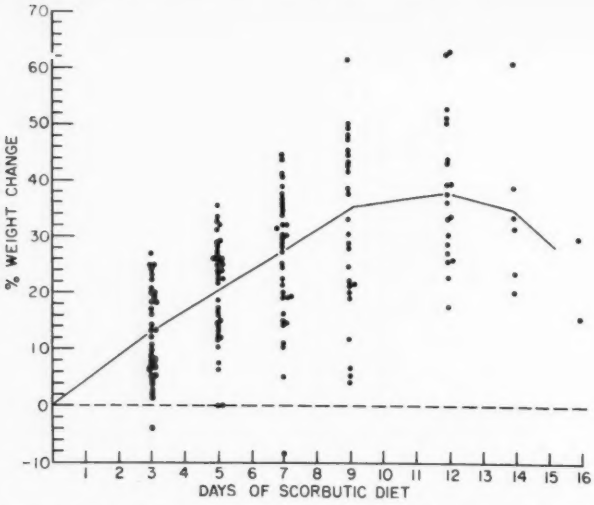
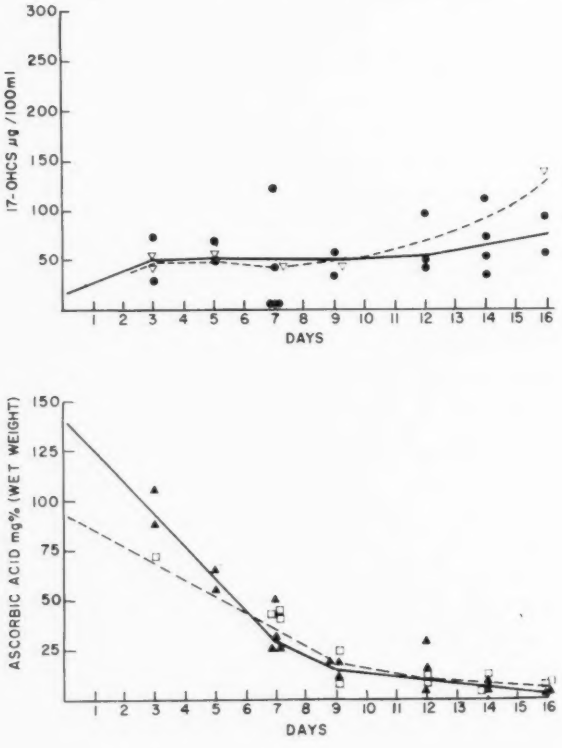


FIG. 1. Changes in body weight following the administration of a Vitamin C deficient diet in normal guinea pigs. The average weight curve is plotted.



at the initiation of the experiment were fed the ascorbic acid deficient diet of Rinehart and Mettier (9). The weights of the animals were recorded every two or three days. Histologic sections of the costochondral and femorotibial (knee) joints of all animals were prepared for the morphologic evaluation of Vitamin C deficiency.

After 3, 7, or 12 days on the deficient diet, the guinea pigs were given a single intravenous injection of C^{14}-labeled *K. pneumoniae* polysaccharide complex (1 mg./100 gm. body weight). The C^{14}-labeled bacterial polysaccharide, prepared by an alkaline-extraction procedure, was non-lethal, non-antigenic, but haptenic (10). Animals were killed by the cervical clamp technique (11) at the second, sixth, fourteenth, and twenty-fourth hours after the injection of the polysaccharide. Plasma and tissue concentrations of C^{14} were determined as described previously (10). Blood, obtained by cardiac puncture in a syringe moistened with commercial heparin solution, was immediately centrifuged and plasma removed. Free 17-OHCS were determined as described by Eik-Nes (12). Ascorbic acid content of the adrenal and liver was determined by the technique of Roe and Kuether (13).

After 7 days on the ascorbic acid deficient diet, guinea pigs were given ACTH intravenously (0.5 I.U./100 gm.) and the plasma 17-OHCS determined at the second, fourth, sixth, ninth and twelfth hours after administration of the tropic hormone.

Guinea pigs maintained on the ascorbic acid deficient diet for 7 days were given cortisol-4-C^{14} intravenously (.0116 μg. or 0.045 μc./100 gm. body weight in 0.1 ml. 0.85% sodium chloride). Groups of four animals were killed at $\frac{1}{2}$, 1, 2, 4, 6, and 9 hours after the injection. Plasma, obtained immediately by centrifugation of heparinized blood, was extracted with methylene dichloride. The aqueous phase was then extracted with butanol. Carbon^{14} from cortisol-4-C^{14} was determined by a windowless counter. The methylene chloride extractable fraction is designated "free" steroids and the butanol extractable fraction is designated "conjugated" steroids. No C^{14} remained in the plasma after the butanol extraction.

RESULTS

Scorbutic Lesions

The guinea pigs on the ascorbic acid deficient diet progressively gained weight until about the twelfth day of the diet (Fig. 1). The few animals which lost weight during this regime were excluded from the experiment. By the seventh day of the scorbutic diet occasional hemorrhages were found about the costochondral junctures and over the femorotibial joints. Histologic examination showed mild "Gerüstmark" beneath the costochondral and tibial epiphyses. The osteoblasts were increased and osteoid tissue was not present at the borders of some spicules of cartilaginous matrix. Between the subepiphyseal spicules, marrow cells were absent and

FIG. 2. Changes in plasma levels of 17-OHCS and adrenal and liver ascorbic acid in normal guinea pigs following the feeding of a Vitamin C deficient diet.

Upper part

●——● plasma levels of 17-OHCS (γ/100 ml.)

▽- - - -▽ bile levels of 17-OHCS (γ/100 ml.)

Lower part

○ ascorbic acid concentrations in the liver (mg.%×10)

▲ ascorbic acid concentrations in the adrenal cortex (mg.%)

some metachromatic material was seen. By the twelfth day of the deficient diet, these changes were more pronounced and eosinophilic, proteinaceous material was present about some fractured cartilaginous spicules.

17-OHCS and Ascorbic Acid

A few days after the initiation of the Vitamin C deficient diet, the plasma 17-OHCS were slightly elevated (Fig. 2). When the plasma 17-OHCS increased, these steroids also appeared in the bile (Fig. 2). The ascorbic acid concentration in the adrenal and liver diminished rapidly during the first 7 days of the deficient diet and more slowly thereafter, reaching very low levels by the twelfth day. Animals with diarrhea and weight loss after 14 to 16 days on the deficient diet had plasma 17-OHCS values over 300 μg./100 ml. Such animals were excluded from this study.

Effect of Administration of Bacterial Polysaccharide and of ACTH

Administration of the bacterial polysaccharide to animals which had

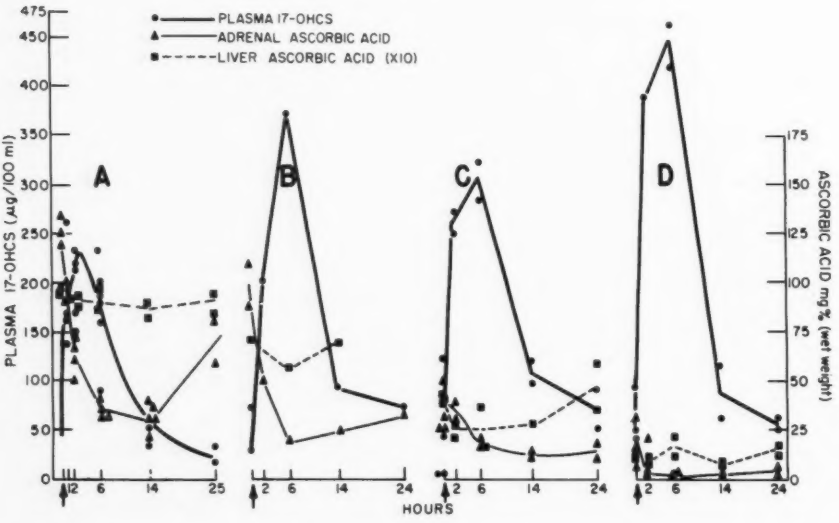


FIG. 3. Plasma levels of 17-OHCS and adrenal and liver ascorbic acid concentrations in normal and scorbutic guinea pigs following the intravenous administration of *K. pneumoniae* polysaccharide (1 mg./100 gm. of body weight).

A: the normal animal

B: animals on a vitamin C deficient diet for 3 days

C: animals on a vitamin C deficient diet for 7 days

D: animals on a vitamin C deficient diet for 12 days

The average curves of plasma 17-OHCS and ascorbic acid concentration in the liver and in the adrenal are plotted in each instance. Arrow indicates when the polysaccharide was given.

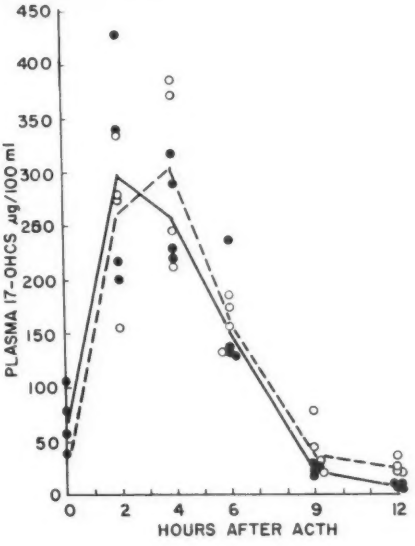


FIG. 4. Plasma levels of 17-OHCS following the intravenous administration of 0.5 I.U. of ACTH per 100 gm. of body weight to normal guinea pigs and guinea pigs who had been on a Vitamin C deficient diet for 7 days.

○- - - - - - - - ○ average curve from normal animals

●———— ● average curve from the ascorbic acid deficient animals

been on the deficient diet for 3 and 7 days evoked a plasma 17-OHCS response of somewhat increased degree and duration (Fig. 3). When the animals were kept on the diet for 12 days, the administration of bacterial polysaccharide gave a plasma 17-OHCS response of somewhat greater magnitude but of similar duration. Regardless of the initial level of adrenal ascorbic acid, the injection of polysaccharide led to an additional decrease in its concentration with a return toward the starting value within 24 hours. A significant fluctuation in the ascorbic acid content of the liver did not appear following the injection of the bacterial polysaccharide. The plasma 17-OHCS response to ACTH in guinea pigs on the deficient diet for 7 days was similar to that in control animals (Fig. 4).

Plasma Clearance of Cortisol-4-C^{14}

When this compound was injected intravenously, it rapidly disappeared from the blood stream (Fig. 5). After 30 minutes only 2.4 to 5.2% remained in the plasma. Both the guinea pigs on the deficient diet for 7 days and the control animals showed a similar exponential loss of free carbon^{14} from cortisol-4-C^{14} over the period of observation.

Within 30 minutes after the injection of labeled cortisol, labeled conjugates appeared in the plasma and were maintained at approximately the same concentration over the 9-hour period of observation (Fig. 6). The only difference in the control and ascorbic acid deficient animals was found during the first 30 minute period. At this time the deficient animals had less conjugated C^{14} than did the controls.

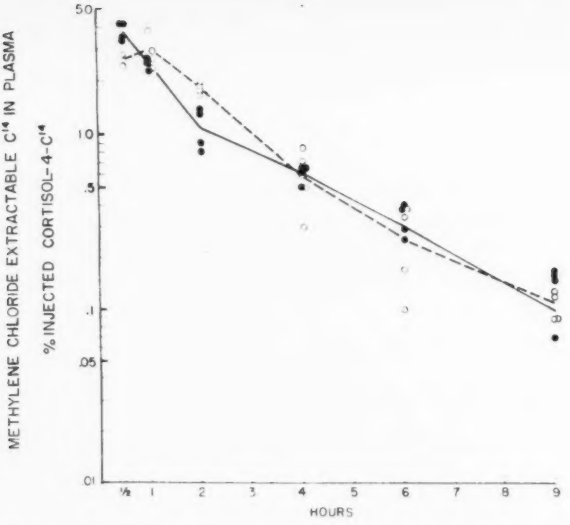


FIG. 5. Methylene chloride extractable C^{14} from the blood plasma of normal animals and animals on a Vitamin C deficient diet for 7 days following the intravenous administration of cortisol-4-C^{14}. The C^{14} concentrations are plotted semilogarithmically against time after steroid administration.

○- - - -○ average curve from normal animals

●————● average curve from scorbutic animals

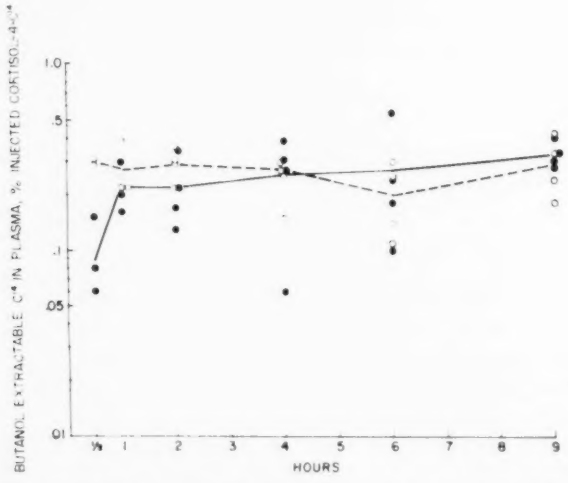


FIG. 6. Butanol extractable C^{14} in plasma from normal animals and from animals fed a vitamin C deficient diet for 7 days following the intravenous injection of cortisol-4-C^{14}. The C^{14} concentrations are plotted semilogarithmically against time after steroid administration.

○- - - -○ average curve from normal animals

●————● average curve from scorbutic animals

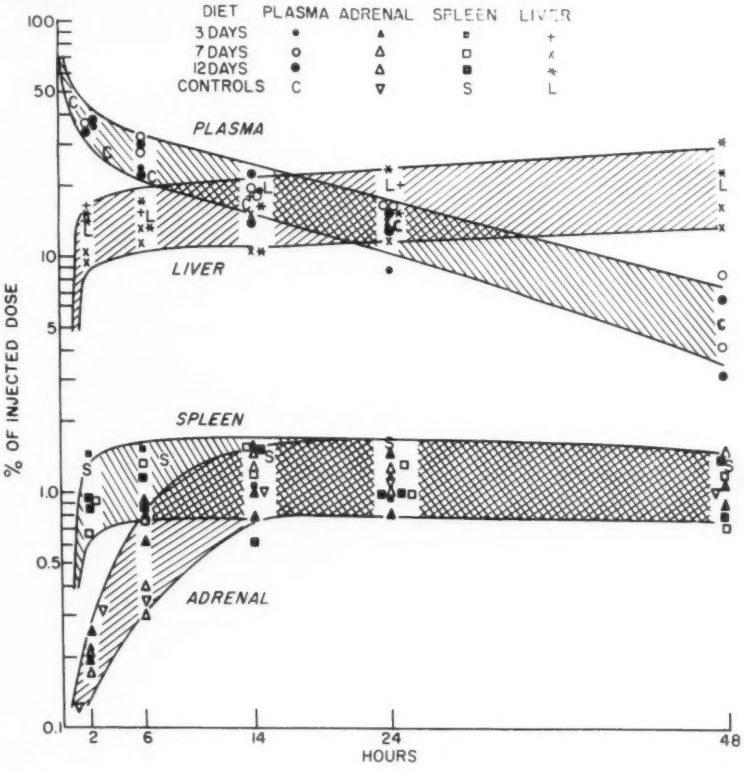


FIG. 7. Distribution of C^{14} from C^{14} containing *K. pneumoniae* polysaccharide in the adrenal gland, the spleen, the liver, and in the blood plasma of normal animals and animals who had been on a Vitamin C deficient diet for different periods of time.

Plasma Clearance and Tissue Distribution of Labeled Polysaccharide

The clearance of C^{14} labeled polysaccharide from blood plasma was not modified by the duration of the ascorbic acid deficient diet (Fig. 7). The rapid uptake of C^{14} by the liver and spleen was similar to that observed in control animals. The slower uptake of C^{14} by the adrenals of normal animals was not modified by ascorbic acid deficiency. The per cent of C^{14} incorporated from the labeled polysaccharide in the thymus, cervical and mesenteric lymph nodes in normal and vitamin C deficient animals was studied but no difference found.

DISCUSSION

The excretion and metabolism of 17-OHCS in the early stages of vitamin C deficiency in the guinea pig may be quite different from that of the advanced stages of vitamin C deficiency characterized by loss of weight, hair,

and fluid. Under such conditions, additional nutritional deficiencies may be expected to create a more complex metabolic disturbance than the lack of ascorbic acid alone.

The present data indicate the ascorbic acid deficient diet leads to a slight elevation in plasma 17-OHCS. In the early phases of ascorbic acid deficiency, Nadel and Schneider (5) observed a decrease in excretion of urinary formaldehydogenic steroids and Clayton and Prunty (3) found a decrease in excretion of 17-ketosteroids in urine. In advanced scurvy a moderate increase in the urinary excretion of formaldehydogenic substances (5) and 17-ketosteroids (3, 4) were apparently accompanied by very high levels of 17-OHCS in the plasma (6). In the present study it has been shown that the clearance of cortisol-4-C^{14} from plasma is not altered in early ascorbic acid deficiency. The data also suggest that the conjugation of this steroid is practially unchanged (Fig. 6). The exponential clearance rate of free cortisol from the plasma in the guinea pig is very similar to that found in man, but the rate of appearance of conjugates is somewhat different (14).

The experimental study of vitamin C deficiency and of scurvy has been hampered by the lack of clear cut correlative criteria. Even the ascorbic acid content of tissues seems to have no direct correlation with the onset of obvious macroscopic lesions indicative of scurvy (15). In the present study, the microscopic evidence of scurvy in the epiphyses appeared within 7 days after the initiation of the ascorbic acid deficient diet. At this time, the ascorbic acid content of adrenal and liver was decreased to 19% and 47% respectively of the original concentration. These animals were still gaining weight.

Animals fed a vitamin C deficient diet for 7 days showed no disturbance in the clearance or conjugation of exogenous cortisol, and the plasma 17-OHCS elevation after ACTH was not modified. The increase in plasma 17-OHCS following the administration of bacterial polysaccharide at the third and seventh days of deficiency was only slightly greater than that of normal animals. In normal animals plasma levels of 17-OHCS from 1–30 γ/100 ml. have been found. Scurvy animals usually had higher levels. Since neither the ACTH response nor the plasma clearance of cortisol was changed at the seventh day of a vitamin C deficient diet, the increase in the control levels of plasma 17-OHCS can only be attributed to a slight increase in circulating ACTH. Whether or not this reflects an increased production of ACTH due to the "stress" of scurvy or decreased catabolism of ACTH in the scurvy animals is unknown.

Oesterling and Long (16) found that the administration of ACTH to guinea pigs which had been on a scorbutic diet for 17 days did not decrease their adrenal ascorbic acid. This may be a characteristic of late scurvy, since in our investigation adrenal ascorbic acid, regardless of initial level, decreased after the injection of bacterial polysaccharide at the third.

seventh or twelfth day on the deficient diet. The rebound of adrenal ascorbic acid in our scurvy animals given polysaccharide was, however, slower than in the normal animal.

The exact role of ascorbic acid in the synthesis and release of 17-OHCS by the adrenals has not been clearly demonstrated. Ascorbic acid may have a regulatory or "braking" role in these processes. In late scurvy the marked plasma 17-OHCS elevation (6) and the increased urinary excretion of formaldehydogenic substances (5) are reduced by the administration of vitamin C. The possible function of ascorbic acid in the cellular hydrogen and electron transport systems has recently been given new emphasis. Haynes and Berthet (17) have proposed that ACTH increases the biosynthesis of adrenal steroids by acting upon phosphorylase. This enzyme acts through glycogen and glucose-1-phosphate to form glucose-6-phosphate, which in turn, maintains the reduced state of triphosphopyridine nucleotide (TPNH). Kersten, Kersten, and Staudinger (18) found on the other hand that swine adrenal mitochondria would influence the hydrogenation of DPNH. Although the mitochondrial fraction contains considerable ascorbic acid, the addition of further ascorbic acid to their *in vitro* system augments the dehydrogenation of DPNH.

While the studies of Schmidt and Staudinger (19) indicate that ascorbic acid, *in vitro*, increases the swine adrenal corticosteroidogenesis, Hayano *et al.* (20) have reported that ascorbic acid, *in vitro*, inhibited the biosynthesis of steroids in cow adrenals.

Following ACTH administration to living animals, the ascorbic acid lost by the rat adrenal enters the blood stream (21). Since ascorbic acid is not synthesized by the adrenal glands in most animals (22), the ascorbic acid must re-enter the adrenal from the blood. Factors regulating the exit and entrance of ascorbic acid in the adrenal gland may therefore reflect modifications in the cellular oxidation-reduction systems important in adrenal steroidogenesis. While the exact role of ascorbic acid is obscure, the present study indicates that moderate ascorbic acid deficiency in the guinea pig, sufficient to produce microscopic changes in the epiphyseal areas but prior to weight loss and sickness, does not significantly modify the elaboration or metabolism of 17-OHCS.

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THE INFLUENCE OF ESTRADIOL AND CORTISOL ON UTERINE HISTAMINE OF THE OVARIECTOMIZED RAT[1,2]

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ABSTRACT

Four hours following a single intravenous dose of estradiol-17β to the ovariectomized rat, uterine histamine concentration was significantly decreased. The decrement was not due to an increase in histaminase activity in the organ. Cortisol given concomitantly did not prevent the estrogen-induced release of histamine, although it did block the uterine hydration response due to estrogen as earlier described from these laboratories. Uterine histamine concentration was restored to control levels within 14 hours following estrogen injection. Uteri taken from intact, untreated animals during those phases of the estrous cycle associated with elevated estrogen secretion exhibited histamine levels which were significantly reduced below those in the quiescent state.

It is proposed that the early accumulation of water, electrolytes and plasma protein following estrogenic stimulation of the uterus are secondary manifestations of the hyperemia and increased capillary permeability resulting from estrogen-induced release of endogenous histamine.

NUMEROUS studies have demonstrated that estrogen administration to the immature or castrated adult rat rapidly induces uterine hypermia (1–3) which is accompanied by uptake of water (4, 5), electrolytes (6–9), and labeled plasma proteins (8) in this organ. The probable functional relationship between estrogen-induced uterine hyperemia, increased capillary permeability and these early correlates of estrogen stimulation has been noted (9–13). The manner in which estrogen elicits the vascular response is unknown. Available data do not support the hypothesis that acetylcholine plays a mediating role in this regard (2, 10, 13–15). On the other hand, in 1924, and thus well before the elucidation of the structure of the ovarian hormones, Robinson and Zondek (16) sought to reproduce the action of crude ovarian extracts in eliciting uterine growth by administration of "nonspecific biogenic amino bodies," among them histamine. These workers noted uterine hyperemia and an increase in mass

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[2] A portion of these data is taken from a thesis submitted by Eugene Spaziani in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of California, January, 1958.

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of this organ following the parenteral injection of a total of 3 and 4 mg. of histamine, respectively, to two infantile guinea pigs. Perhaps because these data could not be confirmed (17, 18) and because of the subsequent establishment of the steroidal structure of the estrogenic hormones by others, this isolated empirical finding was buried. Subsequently, Holden (2) found that histamine administration relieved uterine vasoconstriction produced by a variety of agents and suggested on this basis that histamine may function as a physiological intermediate in the estrogen response. Further investigation of this possibility has not been pursued (cf., however, 19, 20). The experiments reported below, and in a forthcoming publication (20) were designed to test this hypothesis. The present studies demonstrate that the release of uterine histamine is under estrogenic control.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats, 4 to 6 months of age and averaging 300 gm. in weight, were used. In experiments requiring injection of hormones or control solutions, the animals were bilaterally ovariectomized 14 to 16 days earlier. Water and Purina laboratory chow were fed *ad libitum*.

Estradiol-17β was prepared in isotonic saline solution (21) and given in the amount of 0.5 μg. (0.5 ml.)/100 gm. body weight. Cortisol acetate in aqueous suspension (25 mg./ml.) was administered in the amount of 2.5 mg./100 gm. body weight alone or immediately preceding the estrogen. The hormones were given under light ether anesthesia at "0 time," each in a single dose via the saphenous vein; controls received the vehicle alone. At time intervals thereafter, each animal was stunned by a sharp blow at the base of the skull and bled by decapitation. Uterine horns were severed at their juncture and at or near the Fallopian tubes and stripped of extraneous fat and mesentery.

Determination of histamine content

Uterine horns were slit longitudinally, blotted, weighed in pairs on a Roller-Smith balance and placed immediately into a mortar containing 3.5 ml. cold 10% trichloroacetic acid. The horns from 5 rats in any one experimental group were pooled for each determination. In addition, the tissue from a sixth animal in each series was assayed for water content. The procedures employed for both extraction and assay of histamine were essentially those of Barsoum and Gaddum (22) as modified by Code and McIntire (23). The standard employed was histamine dihydrochloride (reagent grade, Eastman). All figures for histamine content in standard and unknown preparations were expressed in terms of histamine base.

Recovery of added histamine

Tissue from untreated castrated rats was used in these determinations. Left and right uterine horns from each of 8–10 donor rats were individually weighed and collected in separate mortars containing 3.5 ml. 10% trichloroacetic acid. This sampling procedure, involving pooling of contralateral uterine horns from the same group of animals, permitted the preparation of two qualitatively homogeneous sets of tissues. To one mortar was added 1.0 μg. of histamine base in 0.05 ml. distilled water, whereas the other received the same volume of water only. Both groups of tissue were then ground and carried through the extraction and assay procedures as above.

Histaminase activity

The volumetric technique of Kapeller-Adler (24, 25) for the estimation of histaminase activity in biological fluids was adapted for analysis of uterine extracts prepared in cold M/15 Sörensen phosphate buffer at pH 7.2. Both horns from 4 animals were pooled for each determination.

Water-soluble nitrogen was determined on aliquots of the tissue extract. Nitrogen analyses of these samples, and of others arising from experimental procedures indicated below, were carried out as described by Szego and White (26).

Uterine DNA

It was thought advisable to obtain an independent measure of the estrogen-induced increase in uterine solids. This was estimated by analysis of total N, a constituent which reflects the significant contribution of protein to the early dry weight increase [apparently by infiltration from the plasma (8)], and relating its concentration to an index of cell numbers. Accordingly, the following determinations were made of total N, and of uterine DNA, a substance which has been demonstrated to undergo no change in the castrated rat within 20 hours of estrogen administration (27–30).

Both uterine horns were taken from 2 rats for each determination, weighed and collected for subsequent grinding with sand in a chilled mortar containing 2 ml. of cold distilled water. One ml. water was added during the 10 minute grinding period and the resulting homogenate transferred to a chilled test tube. The mortar was rinsed with 1 ml. of cold water and the washings added to the tube with mixing. A 2 ml. sample of the whole homogenate was promptly removed to a centrifuge tube containing 2.5 ml. ice cold 10% trichloroacetic acid. Duplicate 0.5 ml. aliquots of the homogenate were also taken for determination of total nitrogen. The sample in trichloroacetic acid was centrifuged at 5° C and further carried through the extraction process for DNA as described by Schneider (31) except that the step involving removal of the phospholipid fraction was omitted. DNA was determined colorimetrically by the method of Dische as modified by Stumpf (32).

RESULTS

Studies on the reliability of the methods employed

The percentage recoveries shown in Table 1 compare favorably with those for blood reported by Code (33).

As supplementary proof that the agent being assayed in uterine extracts was indeed histamine, blocking experiments with antihistamines were performed. The guinea pig ileum segments used for assay were allowed to

TABLE 1. RECOVERY OF HISTAMINE ADDED TO POOLED[1] UTERI FROM OVARIECTOMIZED ADULT RATS

Age of rats (days)	Group I	Group II		μg. hist. recovered per gm. wet wt.	% recovery
	μg. hist. per gm. wet wt.	μg. hist. per gm. wet wt.	μg. hist. added per gm. wet wt.		
195–200	6.56	7.97	1.60	1.41	88.1
200	6.74	8.48	1.65	1.74	105.5
113	5.31	6.89	1.62	1.58	97.5
154–176	5.20	6.52	1.38	1.32	95.7

[1] See text for constitution of pooled samples.

FIG. 1. Inhibition of histamine-induced contractions of guinea pig ileum with Neo-Antergan maleate. Numbers refer to volume (ml.) of solution added. S—histamine standard (0.4 μg. of the base/ml.); U—uterine extract; N—Neo-Antergan maleate (0.1 mg./ml.). See text for experimental details.

contract in turn to additions of histamine standard and of uterine extract. After the usual rinsing, 5 μg. of pyranisamine ("Neo-Antergan") maleate (Fig. 1) or of tripelennamine ("Pyribenzamine") HCl (Fig. 2) were added to the suffusion chamber. Addition of antihistamine was not followed by rinsing. As revealed by the Figures, subsequent addition of extract or standard produced little or no response.

Histamine concentration of the uterus as influenced by steroid hormones

As shown in Table 2, intravenous administration of estradiol resulted 4 hours later in a 24% reduction of uterine histamine concentration on a dry weight basis. Dry weight was calculated for each experimental group from the average water content of uteri from additional animals injected concurrently. The uterine histamine values shown in Table 2 on a fresh weight basis were discounted since the weight increases due to the usual influx of fluid into the uterus following estrogen would preclude meaningful determination of alterations in histamine concentration. The decline from control levels expressed on either a dry or wet weight basis was, however, highly significant by Student's "t" test ($P < 0.001$). Pending further testing and correction for probable uncontrolled variables (see below), these data

FIG. 2. Inhibition of histamine-induced contractions of guinea pig ileum with Pyribenzamine HCl. Numbers refer to volume (ml.) of solution added. ST.—histamine standard (0.8 μg. of the base/ml.); H—uterine extract A—artifact introduced on stopping the drum; P—Pyribenzamine (0.1 mg./ml.).

suggested that a release and disappearance of locally contained histamine had occurred under the influence of estrogen.

Small dry weight increases have been reported from measurements on whole uterus 4-6 hours following a single dose of estrogen (6, 8, 12). Therefore, in order to determine the true decrement in histamine concentration of uteri from estrogen-treated animals it was necessary to assess the extent of any relative increase in solids. Correction factors for dry weight were accordingly derived through two different experimental approaches. One

TABLE 2. THE EFFECT OF ESTRADIOL AND CORTISOL ON THE HISTAMINE CONCENTRATION OF POOLED UTERI FROM ADULT, OVARIECTOMIZED RATS[1]

Treatment	No. of determ's.	% uterine water	μg. hist. base per gm. wet wt.	μg. hist. base per gm. dry wt.
Saline	7	79.6 ± 0.8†	6.92 ± 0.26	33.9 ± 1.3
Estradiol‡	8	85.4 ± 0.6**	3.75 ± 0.37**	25.7 ± 2.5**
Estradiol‡ + cortisol§	4	79.6 ± 0.5	5.75 ± 0.35**	28.2 ± 1.7**
Cortisol§	4	79.5 ± 0.5	7.07 ± 0.60	34.5 ± 2.9

[1] Animals were sacrificed 4 hours after intravenous injection.
** $P < 0.001$ (compared to saline control).
† mean ± standard deviation.
‡ 0.5 μg./100 gm. body weight.
§ 2.5 mg./100 gm. body weight.

TABLE 3. TOTAL NITROGEN AND DNA DETERMINATIONS ON POOLED UTERI TAKEN FROM (a) INJECTED ADULT OVARIECTOMIZED RATS AND (b) INTACT UNTREATED ADULTS IN TWO PHASES OF THE ESTROUS CYCLE

Treatment or condition	No. of determinations	% total nitrogen	% DNA	Mg. N/mg. DNA
(a) Saline control	9	2.47 ±0.06†	0.857 ±0.081	2.91 ±0.33
4 hrs. after estradiol‡	7	1.65 ±0.07	0.535 ±0.070	3.12 ±0.38
14 hrs. after estradiol‡	4	1.98 ±0.09	0.507 ±0.060	3.94 ±0.30
(b) Late metestrus to diestrus	4	2.18 ±0.15	0.285 ±0.022	7.68 ±0.91
Early estrus to estrus	4	2.02 ±0.15	0.304 ±0.011	6.66 ±0.51

† Mean ±standard deviation.
‡ 0.5 μg./100 gm. body weight, I.V.

procedure was based on observations that cortisol prevented estrogen-induced increases in uterine water (12), dry weight (12) and sodium (9). Table 2 reveals that the uterine histamine concentration of the cortisol-treated group was indistinguishable from that of the controls. Table 2 further demonstrates that 4 hours after the concomitant intravenous administration of estradiol and cortisol, a significant decrease in histamine concentration from the control level was evident ($P < 0.001$). In this group, however, the extent of histamine depletion (17% below that of the controls) was not as great as that seen with estradiol alone. The fall in histamine concentration from the control level of 33.9 μg./gm. dry weight to the estrogen plus cortisol level of 28.2 can be regarded tentatively as the decrease due to estrogen alone if this implicit correction for dry weight is used.

The second procedure used for the estimation of a dry weight correction factor was the determination of total nitrogen in uteri treated as above and expression of these values in relation to the DNA content. The ratios of total N to DNA for the control and 4-hour estrogen groups are shown in Table 3, section a. A 7% increase over controls in the N:DNA ratio was noted. This agrees with the dry weight correction derived in the cortisol experiment (*v. s.* and Table 2). However, in both cases the increments in dry weight observed were not statistically significant and might thus be considered maximum values at best.

Histamine concentration determined at the 14-hour interval following estradiol (Table 4) showed a decrease of 34% below the controls. This decline could be accounted for by the increase in dry weight of the organ. (Table 3).

It was of interest to determine whether or not the depletion of histamine observed in the estrogen-treated castrated rat could also be detected during the normal estrous cycle. Accordingly, adult animals were selected on the basis of vaginal smears and separated into two groups, those in early estrus to estrus and those in late metestrus to diestrus. The animals were sacrificed within an hour after the smears were taken. Analysis of the

TABLE 4. HISTAMINE CONCENTRATION OF POOLED UTERI TAKEN FROM (a) ADULT OVARIECTOMIZED RATS INJECTED 14 HOURS EARLIER WITH A SINGLE DOSE OF ESTROGEN AND (b) INTACT UNTREATED ADULTS IN TWO PHASES OF THE ESTROUS CYCLE

Treatment or condition	No. of determ's.	% uterine water	μg. hist. base per gm. wet wt.	μg. hist. base per gm. dry wt.
(a) Saline	7	79.6 ± 0.8†	6.92 ± 0.26	33.9 ± 1.3
Estradiol‡	5	83.0 ± 0.3**	3.82 ± 0.32**	22.5 ± 1.9**
(b) Late metestrus to diestrus	4	82.5 ± 0.6	2.08 ± 0.20	11.9 ± 1.1
Early estrus to estrus	4	81.1 ± 0.9	1.62 ± 0.16	8.5 ± 0.9*

* $P < 0.01$ (compared to late metestrus-diestrus).
** $P < 0.001$ (compared to saline control).
† Mean ± standard deviation.
‡ 0.5 μg./100 gm. body weight, I.V.

early estrus to estrus group revealed a histamine concentration of 8.5 ± 0.9 μg. histamine base equivalents per gm. dry weight in contrast to 11.9 ± 1.1 μg./gm. for rats in late metestrus to diestrus, a difference of 29% ($P < 0.01$, Table 4). No significant difference could be detected between these groups in dry weight as inferred from N to DNA ratios (Table 3, section b).

Uterine histaminase activity

Measurements of the histaminolytic power of uterine homogenates demonstrated that the histamine depletion observed in the uterus 4 hours after estradiol injection was not due to an increased enzymatic destruction of histamine (Table 5).

The titration of the tissue blanks in this method permitted the incidental observation to be made that there was consistently less endogenous his-

TABLE 5. HISTAMINASE ACTIVITY OF POOLED UTERINE TISSUE TAKEN FROM ADULT OVARIECTOMIZED RATS 4 HOURS AFTER A SINGLE DOSE OF ESTROGEN[1]

Saline control				Estradiol treated			
No. of determ's.	% water-soluble N	P.U.[2] per gm. fresh tissue	P.U. per mg. N	No. of determ's.	% water-soluble N	P.U. per gm. fresh tissue	P.U. per mg. N
6	0.956 ± 0.035†	245 ± 42	25.7 ± 4.9	5	0.855 ± 0.017	212 ± 33	24.8 ± 3.8

[1] 0.5 μg./100 gm. body weight, I.V.
[2] Permanganate units. One P.U. represents that amount of enzyme which after aerobic incubation for 24 hours at 37° C and pH 7.2, in the presence of 1 ml. of 0.067% indigocarmine takes up 0.1 ml. of 0.002 N $KMnO_4$ (24, 25). Under these conditions, 1 P.U. corresponds to the destruction of 0.46 μg. of histamine/hr. (24).
† Mean ± standard deviation.

tamine substrate present in the estrogen-treated groups than in the controls ($\Delta KMnO4 = 0.52 \pm 0.18$ ml.). This finding supports the direct observations on the capacity of estrogen to evoke uterine histamine depletion described above.

DISCUSSION

This histamine concentration of a number of tissues in various mammalian species has been tabulated by Feldberg (34). Values for the rat ranged from more than 40 μg./gm. of fresh tissue in skin of the feet to less than 1

μg./gm. in liver and spleen. The figure of approximately 7 μg./gm. found in the present studies for the uterus of the ovariectomized rat falls within the range determined for stomach mucosa, lungs and heart in the rat, and is similar to the value of 11 μg./gm. and to the range of 13–22 μg./gm. reported by Mongar and Schild (35) and by Effkemann and Werle (36), respectively, for the uterus of intact guinea pigs.

The corrected values for the decrease in histamine concentration observed under short term estrogen treatment could not be accounted for on the basis of an increase in histaminase activity determined under the same conditions. This is consistent with existing reports of longer term experiments in which estrogen was seen to have no effect on the histaminase content of the uterus in the ovariectomized rat (37) or rabbit (38). Furthermore, the histamine depletion cannot be considered a secondary consequence of estrogen-induced uterine hydration through disruption of the osmotic relations of histamine-containing cells, since cortisol given with the estrogen prevented water uptake but had no effect on the accompanying loss of the amine.

In contrast to the findings at 4 hours, analyses at 14 hours failed to show an alteration of histamine concentration from control levels after the dry weight correction was applied. It appears, then, that within 14 hours histamine repletion is effected.

Preliminary studies in the intact animal showed that uteri taken in the phase of high estrogen secretion (early estrus-estrus) contained approximately 29% less histamine per gm. dry weight (uncorrected) than the organs undergoing cyclic involution. The N:DNA ratios indicated no significant relative change in dry weight between these periods under the conditions studied. Thus, the depletion of histamine in the early estrus to estrus period of the cycle, presumably under the influence of the prevailing high blood estrogen level may be considered to be real rather than apparent. At both stages of the cycle examined, the uterine histamine concentration was strikingly lower than in either the ovariectomized control or in the castrate under acute estrogen treatment. Since these data were expressed on the basis of dry weight most, if not all, of the difference seen in histamine concentration between uteri from intact animals and those of treated or untreated castrated rats can be attributed to a loss in total weight of the uterus which occurred after ovariectomy. The finding of no significant differences in water content of the tissue for the stages of the estrous cycle has been observed previously (39).

The data presented are interpreted to mean that an early consequence of estrogen action in the uterus is the release of histamine in the organ. Further, it is postulated that histamine thus liberated brings about the local hyperemic reaction, earlier noted by others (1–3), which is in turn responsible for initial phases of edema formation which is well established in the uterus within 3 hours (9) following estrogen administration.

Corroborative evidence includes the observations that a histamine releasing agent, or histamine itself, when injected locally, mimics the action of estrogen with regard to eliciting hyperemia and hydration in the organ (19, 20). Moreover, blockade of estrogen action in these respects can be achieved with certain histamine antagonists (19, 20).

Paton (40) has summarized the theories advanced to explain the mechanism(s) of histamine release or mobilization from tissues by histamine "liberating" compounds of various types [cf., also, discussions by McIntire (41) and Ungar (42)]. These theories variously propose that histamine releasers act by: (a) inhibition of histaminase, (b) acceleration of histidine decarboxylase, (c) activation of proteolytic enzymes which, in turn, mobilize histamine from the tissue, (d) displacement of histamine base from acidic intracellular binding sites by virtue of the releaser's greater affinity for those sites (a theory made attractive by the fact that many releasers studied are themselves simple bases) or, (e) lytic or other action on the surface of the cell or its particulate components. Mechanism (e) is derived from evidence that *in vivo* histamine depletion can be accomplished by surface-active materials including saponin and bile salts (40, 41).

In considering the means by which estrogens may promote histamine release in the uterus, theory (a) above may be regarded unlikely on the basis of the present findings. Theory (b) does not appear applicable to the data reported in which liberation of histamine was accompanied by disappearance from the tissues. The fact that the estrogens are acidic in the physiological pH range (43) militates against mechanism (d). Theories (c) and (e) remain to be investigated. The capacity of estrogenic materials to alter the surface properties of protein monomolecular layers (44), together with the known affinity of these and other steroids for specific proteins (13, 45, 46) would indicate that exploration of mechanism (e) may prove fruitful.

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THE STABILITY OF NATURAL AND SYNTHETIC NEUROHYPOPHYSIAL HORMONES *IN VITRO*[1]

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ABSTRACT

The spontaneous decomposition of synthetic and natural neurohypophysial hormones in aqueous buffer solutions over a pH range of 3.0–8.5 at room temperature has been studied. Maximal stability for crude, highly purified and synthetic arginine vasopressin preparations was found at pH 3.0, 5.0 and 8.5. The decay proceeded most rapidly at pH 7.5 in phosphate buffer. The stability of arginine vasopressin at pH 7.5 was higher in diethylbarbiturate than phosphate buffer solution. Crude synthetic lysine vasopressin with low initial activity was most stable at pH 3 and 5. It was found to undergo less decomposition in *phosphate* buffer than the arginine congener. The highly purified and synthetic arginine vasopressin preparations underwent decomposition more rapidly than crude posterior pituitary extracts. The ratios of the antidiuretic, vasopressor and milk-ejection potencies of arginine vasopressin remained constant although the potencies of the solutions fell.

The velocity of decomposition of crude natural and highly purified synthetic oxytocin was found to be similar. Both preparations possessed a maximal stability at pH 3. Variations in the hydrogen ion concentration from 5 to 8.5 influenced the rate of decay only to a moderate extent.

The stability of endogenously liberated ADH in plasma was not appreciably lower than that of purified arginine vasopressin in a phosphate buffer solution at 2° C and at the same pH. Oxytocin dissolved in canine plasma underwent inactivation considerably faster than the control in phosphate buffer at similar pH but at room temperature. At 2° C, fifty per cent of activity of oxytocin in plasma was lost in 40 hours.

BEFORE the elucidation of the structure of oxytocin and vasopressin (antidiuretic hormone) by du Vigneaud and his associates, experiments dealing with the stability of the neurohypophysial hormones were undertaken chiefly to furnish information which could help in clarifying their chemical nature. It was shown that the oxytocic activity of pituitary

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extracts was rapidly destroyed by treatment with strong acids and strong alkali (1, 2, 3) and that the disappearance of activity was characteristic of a single substance decomposing according to the law for a monomolecular reaction (4). Further work indicated that the hydrogen-ion concentration rather than the acid used determined the stability of the oxytocic principle and that its inactivation by reducing agents (5, 6) was probably due to reduction of a disulfide bond in the oxytocin molecule. The effect of temperature upon the velocity of decomposition was found to be in satisfactory agreement with Arrhenius' equation (7, 8, 9); the electrolyte concentration of the solution was considered to be non-contributory to this effect. Purification of posterior pituitary extracts was not found to lessen the stability of oxytocin (9). Gaddum (7) has published a nomogram in which the loss of activity of oxytocin is related to time, temperature and hydrogen-ion concentration.

Compared with the considerable information concerning the fate of dissolved oxytocin our knowledge of the stability of the antidiuretic hormone or vasopressin was meager until the work of Heller (10) was published. Employing a pH range from 0.57 to 10.0 at 99° C he demonstrated a maximal stability at pH 3.0 for the antidiuretic hormone (ADH) and vasopressin, which at that time were often considered to be separate entities. The antidiuretic hormone, however, appeared to be the more stable in the alkaline range, as antidiuretic assays of extract heated at pH 8.0 or 10.0 indicated about 10 times greater potency than was disclosed by vasopressor assays. Heller's technics were reviewed by Fraser (11) who demonstrated that partially alkali-inactivated posterior lobe preparations gave higher ADH values if subcutaneous rather than intravenous injections were used. Unfortunately, Fraser's own assay technics were of limited accuracy.

The recent availability of highly purified natural and synthetic neurohypophysial hormones as well as the improvement of methods used for their estimation made possible a more reliable and systematic investigation of their stability. In this study the problem of spontaneous decomposition was approached from a practical point of view, such as the situations encountered during the isolation, concentration, assay and storage of these hormones. The influence of the buffer system used, as well as the degree of purity of the preparation, became of particular interest. Attention was also focused on the behavior of the synthetic octapeptides and a comparison was made between lysine and arginine vasopressions. Another aspect of hormone activity which was studied *in vitro* and is reported here was the stability of endogenously liberated vasopressin (ADH) in plasma.

MATERIAL AND METHODS

The preparations studied were Posterior Pituitary Reference Standard U.S.P. (0.4 U./mg., containing according to pharmacological criteria only arginine vasopressin), highly purified natural arginine vasopressin (400 U./mg.), partially purified synthetic arginine vasopressin (175 U./mg.), an early sample of partially purified synthetic lysine

vasopressin (36 U./mg.) and highly purified synthetic oxytocin (500 U./mg.). The purified natural and synthetic preparations were kindly supplied by Dr. Vincent du Vigneaud. Solutions with hormone concentrations varying from 50 to 200 mU./ml. were prepared in 0.9% aqueous sodium chloride containing buffer and were stored at room temperature (18°–24° C) in tightly stoppered 20 ml. test tubes. A crystal of thymol was added to each tube as a preservative. Acetic acid as a 0.3% solution, and acetate (0.1 *M*), phosphate (0.1 *M*) and diethylbarbiturate (0.035 *M*) buffers were used at pH levels of 3, 5, 6.5, 7.5 and 8.5 respectively. At pH 7.5 both phosphate and diethylbarbiturate buffers were employed. The hydrogen-ion concentration of the solutions was tested occasionally and showed only negligible changes over a period of two months.

The assay methods employed have been described previously (12). For the study of the stability of endogenous ADH, dogs were prepared under pentobarbital sodium anesthesia and given artificial respiration. The neurohypophysis was stimulated by morphine sulfate (1 mg./kg. by intracarotid injection) and blood samples were taken from external and internal jugular veins the extracerebral branches of which had been previously ligated.

RESULTS

Effect of pH and buffer solution on the stability of crude, purified and synthetic vasopressins. Tables 1 and 2 summarize the data obtained during 2 months from studies of various ADH preparations divided into four groups: a) Posterior Pituitary Reference Standard, U.S.P., b) highly purified arginine vasopressin, c) synthetic arginine vasopressin and d) synthetic lysine vasopressin.

The rate of decomposition is expressed as the ratio of assayed value after a given time interval to the original potency. From the decay curves, which differed among the highly purified preparations but were less variable within the series of U.S.P. Standard solutions, most points as listed in Tables 1 and 2 had to be obtained graphically as assays could not always be carried out at the same time-intervals. For the interpretation of these results it must be remembered that the accuracy of either the vasopressor or antidiuretic assay will not exclude errors of ±20%. It is apparent that the spontaneous decomposition of either purified or crude vasopressin preparations proceeded most rapidly at pH 6.5 and 7.5. At hydrogen-ion

TABLE 1. THE STABILITY OF NATURAL ARGININE VASOPRESSIN IN AQUEOUS BUFFER SOLUTIONS AT ROOM TEMPERATURE

	Purified arginine vasopressin[1]						U.S.P. reference standard[2]						
pH buffer	3.0 —	5.0 acet.	6.5 phos.	7.5 phos.	7.5 DEB	8.5 DEB	pH buffer	3.0 —	5.0 acet.	6.5 phos.	7.5 phos.	7.5 DEB	8.5 DEB
days							days						
	1.00	1.00	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00
2	0.98	0.99	0.95	0.79	0.95	0.99	2	0.96	0.96	0.95	0.90	0.97	1.06
4	1.10	0.94	0.86	0.74	0.83	0.94	4	0.97	0.94	0.92	0.84	0.95	1.00
6	1.05	0.90	0.81	0.66	0.79	0.90	6	0.96	0.90	0.89	0.78	0.92	0.94
8	0.99	0.93	0.76	0.62	0.75	0.94	8	0.97	0.90	0.82	0.73	0.89	0.93
10	0.96	0.96	0.70	0.57	0.72	0.97	10	0.97	0.90	0.80	0.71	0.85	0.92
12	1.01	0.96	0.65	0.48	0.71	0.97	12	0.97	0.90	0.77	0.67	0.81	0.91
14	1.06	0.96	0.61	0.39	0.71	0.96	14	0.97	0.89	0.73	0.64	0.78	0.89
28	1.05	0.80	0.20	0.12	0.33	0.74	28	1.00	0.88	0.60	0.46	0.60	0.80
62	0.75	0.35	—*	—*	—*	0.38	62	0.76	0.90	—*	—*	—*	0.68

Note: DEB = diethylbarbiturate.
* No activity detected.
[1] 400 U./mg.
[2] 0.4 U./mg.

TABLE 2. THE STABILITY OF SYNTHETIC ARGININE AND LYSINE VASOPRESSINS IN AQUEOUS BUFFER SOLUTIONS AT ROOM TEMPERATURE

	Arginine vasopressin[1]							Lysine vasopressin[2]						
pH buffer	3.0 —	5.0 acet.	6.5 phos.	7.5 phos.	7.5 DEB	8.5 DEB	pH buffer	3.0 —	5.0 acet.	6.5 phos.	7.5 phos.	7.5 DEB	8.5 DEB
days							days						
	1.00	1.00	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00
2	1.01	0.98	0.93	0.90	1.02	1.02	2	0.98	0.99	0.96	1.00	1.04	1.00
4	1.02	0.97	0.87	0.80	0.95	1.01	4	0.96	0.99	0.93	0.98	0.97	1.00
6	1.02	0.96	0.80	0.70	0.88	0.99	6	0.95	0.99	0.90	0.96	0.90	1.00
8	1.03	0.95	0.74	0.63	0.80	0.98	8	0.96	0.99	0.88	0.91	0.88	0.99
10	1.01	0.88	0.70	0.57	0.79	0.95	10	0.97	1.00	0.85	0.85	0.85	0.97
12	1.00	0.82	0.65	0.52	0.77	0.93	12	0.98	1.00	0.82	0.80	0.83	0.96
14	0.98	0.76	0.62	0.50	0.72	0.90	14	0.99	1.01	0.80	0.75	0.80	0.94
21	0.93	0.67	0.50	0.42	0.63	0.85	21	0.93	1.00	0.70	0.63	0.72	0.83
28	0.87	0.64	0.37	0.25	0.52	0.75	28	0.87	0.90	0.60	0.53	0.61	0.66
45				0.05									
62	0.88	0.62	—*	—*	0.22	0.52	62	0.78	0.79	0.32	0.24	0.31	0.23

Note: DEB = diethylbarbiturate.
* No activity detected.
[1] 175 U./mg.
[2] 36 U./mg.

concentrations represented by these pHs there was a considerable discrepancy between the stability of crude and highly purified natural arginine vasopressin preparations; the latter decomposed more rapidly. (It should be mentioned that one purified preparation (508123) deteriorated very rapidly. This highly purified arginine vasopressin in a phosphate buffer solution at pH 7.5 lost about 50% of its activity in 3 days.) The effect of the two buffer solutions used at pH 7.5 is of interest. Throughout the experiment the groups containing arginine vasopressin showed a significantly reduced rate of decomposition in 0.035*M* diethylbarbiturate compared with that in phosphate buffer solution at the same pH.

In a comparison of the rates of decay of synthetic arginine and lysine vasopressins certain differences were encountered (see Table 2). Lysine vasopressin, although as stable as the arginine compound in an acid medium, lost less activity in the pH range from 6.5 to 7.5 in phosphate buffer. Whether or not this discrepancy was due to the cruder nature of this early partially purified synthetic lysine vasopressin preparation remains to be elucidated.

Comparison of the stability of antidiuretic, vasopressor and milk-ejection potencies of the U.S.P. Standard preparation and highly purified arginine vasopressin. The relationship between the rates of decay of the antidiuretic and vasopressor potencies was studied with highly purified arginine vasopressin and the U.S.P. Posterior Pituitary Standard. Simultaneous determinations by both assay methods were carried out with solutions at pH 3, 5, 6.5, 7.5 and 8.5 during 2–42 days. The data obtained are presented in Table 3. Antidiuretic and vasopressor potencies are expressed as ratios of the assayed values to the original potency. The results are listed in the order of the degree of decomposition. The first four columns indicate the difference in the solutions used with respect to the source of ADH, the pH, the buffer solution and the elapsed time. In general there was a close agreement between

TABLE 3. COMPARISON OF ANTIDIURETIC AND VASOPRESSOR POTENCIES OF ARGININE VASOPRESSIN UNDERGOING DECOMPOSITION

Preparation	pH	Buffer	Time in days	Antidiuretic potency	Vasopressor potency
U.S.P. Std.	3.0	—	42	1.01	0.98
U.S.P. Std.	3.0	—	28	1.00	1.00
Purif. Vp.	3.0	—	2	0.91	0.96
Purif. Vp.	8.5	diethylbarb.	7	0.91	0.79
U.S.P. Std.	5.0	acetate	28	0.84	0.88
Purif. Vp.	5.0	acetate	23	0.81	0.80
Purif. Vp.	3.0	—	7	0.79	0.84
Purif. Vp.	7.5	diethylbarb.	14	0.64	0.72
U.S.P. Std.	6.5	phosphate	27	0.64	0.56
U.S.P. Std.	7.5	diethylbarb.	27	0.60	0.60
Purif. Vp.	7.5	diethylbarb.	23	0.56	0.46
U.S.P. Std.	7.5	phosphate	27	0.40	0.56
Purif. Vp.	6.5	phosphate	23	0.35	0.36
U.S.P. Std.	5.0	acetate	42	0.34	0.34
				mean = 0.63	0.63

the corresponding values obtained in the antidiuretic and vasopressor assays. To extend these observations an additional experiment using the U.S.P. Standard in a highly alkaline (pH 10.3) medium was undertaken. After one hour in a boiling water bath the loss of the vasopressor and antidiuretic activities was identical (90% loss in the antidiuretic and 87% loss in the vasopressor assay). These results indicate that similar losses of antidiuretic and vasopressor activities occur in a vasopressin solution undergoing decomposition; the parallelism of this decline is unaffected by variations in the hydrogen-ion concentration or differences in buffer solutions.

The decay of the milk-ejection potency of vasopressin was studied with highly purified arginine vasopressin. Solutions were prepared in buffer solutions at pH 3, 6.5, 7.5 and 8.5. Milk-ejection and vasopressor activities were determined at intervals during 34 days. The loss of activity measured by the vasopressin assay ranged from 0 to 90%. As the milk-ejection potency of the ADH is about 17% in terms of U.S.P. Standard (12, 13), this value was used to compare the milk-ejection/vasopressor ratios obtained. In no instance was a significant deviation observed. The mean milk-ejection potency computed from 14 determinations was 17.7% of the respective values found by the vasopressor assay (see Table 4). The agreement satisfactorily demonstrated that the decomposition of vasopressin leads to a parallel loss of both the milk-ejection and vasopressor potencies.

The stability of synthetic and crude natural oxytocin. The stability of oxytocin was investigated according to the plan described above for vasopressin using the same hydrogen-ion concentrations and buffer solutions. Owing to the limited availability of assay animals the determination of oxytocin in terms of milk-ejection potency could not be carried out as frequently as that reported for ADH. However, this did not represent a handicap as a relatively slow rate of decomposition for oxytocin throughout the pH range employed was apparent from the beginning of the investi-

TABLE 4. COMPARISON OF MILK-EJECTION AND VASOPRESSOR POTENCIES OF HIGHLY PURIFIED ARGININE VASOPRESSIN AT VARIOUS STAGES OF DECOMPOSITION

pH	Buffer	Time in days	Per cent of initial vasopressin activity	ME/VP*
3.0	—	4	97	0.14
3.0	—	1	96	0.16
7.5	DEB	4	92	0.19
8.5	DEB	4	90	0.24
8.5	DEB	34	90	0.14
3.0	—	11	82	0.11
3.0	—	14	80	0.14
8.5	DEB	14	78	0.18
6.5	phosphate	4	62	0.21
7.5	DEB	14	60	0.18
7.5	phosphate	4	46	0.24
6.5	phosphate	14	40	0.16
7.5	phosphate	14	25	0.24
7.5	phosphate	34	11	0.15
				Mean = 0.177

Note: DEB = diethylbarbiturate.

* $\frac{\text{Milk-ejection potency}}{\text{Vasopressor potency}}$

gation. The results are summarized in Figure 1.

The maximal stability of synthetic oxytocin like that of vasopressin was found to be at pH 3 in 0.3% acetic acid. Over a period of 4 weeks there was

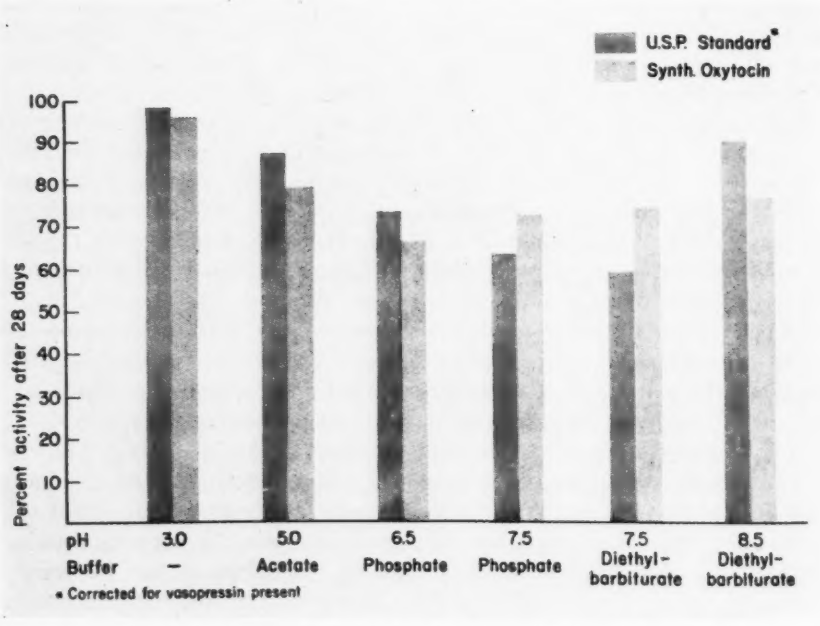

FIG. 1. The stability of crude natural and highly purified synthetic oxytocins in aqueous buffer solutions after 4 weeks at room temperature.

no detectable loss of activity in either the highly purified synthetic preparation or the crude U.S.P. Standard. The decomposition of the synthetic oxytocin over the remaining pH range showed a satisfactory uniformity and was indistinguishable within the error of assay. The mean survival of activity after the 4-week period at pH 5, 6.5 and 7.5 was about 75%. The loss of the milk-ejection potency corrected for vasopressin in the U.S.P. Standard preparation followed a similar pattern. However, an accelerated rate of decay appeared to have occurred at pH 6.5 and 7.5 in phosphate and diethylbarbiturate buffers. At pH 5.0 and pH 8.5, the mean loss of activity after 33 days was about 15%.

Stability of endogenously liberated vasopressin in plasma. The lack of our knowledge regarding the state of the *circulating* hormone as well as its low concentration led to experiments on the survival of endogenous hormone in plasma. In a preliminary investigation the stability of added hormone (purified arginine vasopressin or U.S.P. Standard) in a low (2 mU./ml.) concentration was explored. There was no detectable difference at 2° C in the rate of decay between either type of preparation, and the antidiuretic activity was preserved equally well in plasma and in the control solution of isotonic sodium chloride. Practically no loss of activity could be demonstrated within the first 48 hours although the pH of the plasma was about 7.8 and that of the saline was less than 7 (Fig. 2).

Different results were obtained in a similar study with oxytocin. U.S.P. Reference Standard in a concentration of 25 mU./ml. in canine plasma at

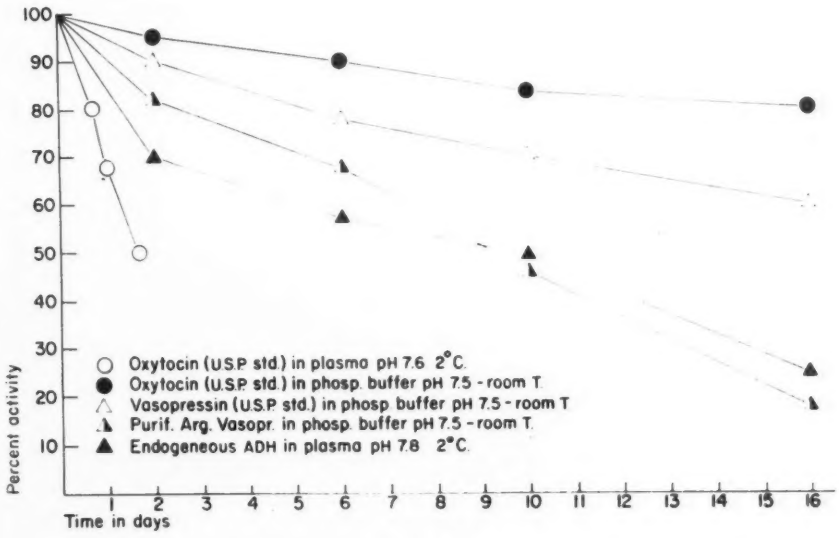
100
90
80
70
60
50
40
30
20
10
Percent activity
Oxytocin (U.S.P. std.) in plasma pH 7.6 2°C.
Oxytocin (U.S.P. std.) in phosp. buffer pH 7.5 - room T.
Vasopressin (U.S.P. std.) in phosp. buffer pH 7.5 - room T.
Purif. Arg. Vasopr. in phosp. buffer pH 7.5 - room T.
Endogeneous ADH in plasma pH 7.8 2°C.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time in days

FIG. 2. The stability of neurohypophysial hormones in plasma and in aqueous phosphate buffer solution.

2° C was used. The oxytocic activity was measured by the milk-ejection assay as described above. The results are presented in Figure 2 and a greatly accelerated loss of activity in plasma is apparent.

Six experiments were conducted to obtain sufficiently large amounts of endogenously-liberated antidiuretic hormone for a further study of stability. Blood samples from donor dogs were taken immediately after the stimulation of the neurohypophysis by the intracarotid injection of morphine sulfate in a dose of 1 mg./kg. over a period of 2 minutes. The concentration of vasopressin in the internal and external jugular venous blood, measured by its intravenous antidiuretic action, showed considerable variations from experiment to experiment. The highest antidiuretic value, 340 mU./100 ml. of plasma, was observed in a sample from the internal jugular vein (blood flow 1 ml./min.) after the third consecutive stimulation. The maximal concentration in plasma obtained from the external jugular vein (blood flow 20 ml./min.) was 80 mU./100 ml. Low hormone concentrations in conjunction with the limited amounts of plasma available led occasionally to considerable difficulty in the performance of accurate assays. However, a general trend in the loss of activity could be clearly demonstrated.

The data obtained from a representative experiment in which the stability of endogenous vasopressin in plasma kept at 2° C was determined are shown in Figure 2. The antidiuretic potency present at a given time is expressed as the fraction of the original concentration. The absolute amount of vasopressin present at the beginning of the experiment was 28 mU./100 ml. of plasma. The pH of the sample throughout the observation period was approximately 7.8. To compare the velocity of decompositon of the endogenously liberated hormone with that of purified arginine vasopressin, the decay of the latter in a phosphate buffer solution of pH 7.5 is presented in the same figure. In general it can be stated that the stability of endogenous vasopressin in plasma is relatively high despite the low concentration of the hormone present and the unfavorable pH of the medium.

DISCUSSION

Although the elucidation of the chemical structure of the neurohypophysial octapeptides has been accomplished, our knowledge of the pathways involved in their spontaneous decomposition remains meager. Experimentally-induced inactivation by acid hydrolysis, reduction of the disulfide linkage or enzymatic cleavage of the peptide bonds has shed some light on the numerous possibilities.

The comparative study of the loss of antidiuretic, vasopressor and milk-ejection potencies of highly purified arginine vasopressin has shown identical rates of decline of activity measured by any of the three methods of bio-assay employed. A parallel loss of vasopressor and oxytocic activity has also been reported for a solution of highly purified arginine vasopressin

(14). Our observations support the concept that the portion of arginine vasopressin, which survives at any of the hydrogen-ion concentrations investigated, is unchanged with respect to its activities. This view is supported by the fact that related octapeptides when biologically active do not possess ratios of activities like those of oxytocin or vasopressin (15). Our experiments failed to demonstrate any appreciable difference in the stability of oxytocin in the pH range of 5 to 8.5 in contrast with the experiments of others. However, it must be emphasized that differences in the design of the experiments render a comparison difficult. Dettelbach (16) concluded that vasopressin in a concentration of 0.5 mu./ml. in aqueous solution at room temperature underwent very rapid inactivation especially at a pH above 4; the soltuions were not buffered and did not contain a preservative. The interesting finding of the greater stability of arginine vasopressin at pH 8.5 in comparison with pH 6.5 or 7.5 is also not in agreement with previous reports.

No explanation at present can be offered for the discrepancy in the rate of decomposition of arginine vasopressin in phosphate and diethylbarbiturate buffers at pH 7.5.

Acknowledgments

We are indebted to Professor Vincent du Vigneaud for gifts of natural and synthetic neurohypophysial hormones.

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THE ANTIDIURETIC POTENCY OF ARGININE AND LYSINE VASOPRESSINS IN THE PIG WITH OBSERVATIONS ON PORCINE RENAL FUNCTION[1]

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ABSTRACT

In the dog intravenous arginine vasopressin is a much more potent antidiuretic agent in terms of its vasopressor activity than lysine vasopressin. Arginine vasopressin appears to be the antidiuretic hormone of the dog and many other mammals whereas lysine vasopressin is the antidiuretic hormone of the pig. In the pig lysine vasopressin is as potent if not more potent than arginine vasopressin when either hormone is administered to unanesthetized animals by intravenous injection.

Renal function studies were also performed in the pig which can excrete about 20 per cent of the glomerular filtrate during water diuresis. The glomerular filtration rate (C_{IN}), the renal plasma flow (C_{PAH}) and the maximal transfer of para-aminohippurate ($T_{m_{PAH}}$) are all much higher in the pig than in most other mammals. The clearance of endogenous creatinine was lower than that of inulin.

ARGININE vasopressin was identified by Turner, Pierce and du Vigneaud (1) as the vasopressin of the posterior pituitary of oxen whereas the vasopressin of hog glands was shown to be lysine vasopressin (Popenoe, Lawler and du Vigneaud (2)). These vasopressins were later carefully compared pharmacologically by van Dyke, Engel and Adamsons (3). Doses with equal vasopressor effects in anesthetized rats were equally potent in raising the blood pressure of dogs, in raising the milk-ejecting pressure in the breast of rabbits, in lowering the blood pressure of fowls and in inhibiting diuresis after *subcutaneous* injection in rats. The striking difference between the two vasopressins was revealed by experiments testing their antidiurectic potency after *intravenous* injection in trained, unanesthetized dogs. By this assay, arginine vasopressin was about six times as potent as lysine vasopressin. Inasmuch as lysine vasopressin appears to be

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secreted only by the pig neurohypophysis, it is of considerable interest to determine the intravenous antidiuretic potency of the two vasopressins in pigs. The experiments which were performed for this purpose are reported here. In addition observations on renal function in the pig are recorded.

MATERIAL AND METHODS

Highly purified natural arginine vasopressin and synthetic lysine vasopressin, prepared in the College of Medicine of Cornell University were kindly provided by Professor V. du Vigneaud. Vasopressor potency in comparison with International Standard Posterior Pituitary Powder was determined repeatedly by intravenous injection in rats during the experiments as well as after their completion. No significant deterioration occurred. All the doses used in pigs were based upon vasopressor standardization.

All the experiments were performed with four female "miniature" pigs purchased from the Hormel Institute of Austin, Minnesota. During the experiments, the weight of the pigs varied between 14.2 and 31.3 kg.

Preliminary experiments in which ethanol or chloralose was used as an anesthetic were unsatisfactory because aspiration of orally-administered fluid frequently occurred. It was soon discovered that the pigs could be readily trained to lie quietly with gentle restraint. All the results were obtained in trained unanesthetized pigs. For hydration the initial priming dose of water, about 1,000 ml. for pigs weighing 15 to 20 kg., was given by stomach tube to pigs restrained in a dorsal recumbent position. Thereafter, owing to the very rapid flow of urine, large volumes were administered every 45 to 70 minutes to maintain the load of administered but unexcreted water at about 1,000 ml. or more before each injection of vasopressin. For example, in Experiment A of Figure 1, the total dose of water including the priming dose of 1,000 ml. was 3,700 ml.; in Experiment B, the total dose of water was 4,350 ml. divided into five doses. After the initial hydration a Malecot indwelling catheter was inserted into the bladder where it remained throughout the experiment. After each collection the bladder was washed with 10 ml. of water which was added to the urine of that period.

All injections of arginine or lysine vasopressin were made intravenously. In the earlier experiments a needle attached to polyethylene tubing was placed in the lateral malleolar vein. Subsequently a modification of the method of Carle and Dewhirst (4) for obtaining blood from the anterior vena cava of swine was used. A thin-walled 18 gauge needle, $1\frac{1}{2}$ inches long, was introduced through the skin of the neck about one centimeter cephalolaterad to the tip of the cariniform cartilage, and was directed in a posterior, medial and caudal direction until it lay within and under the arch of the first ribs. A slight further advance placed the needle in the anterior vena cava. As soon as blood was obtained the syringe was removed and polyethylene tubing (No. 50) was introduced through the needle a distance of 12 to 20 cm. This placed the tubing well down into the vena cava or the right atrium. The tubing thus could be used for injections of hormones or for infusions. If blood samples were concomitantly desired, a second polyethylene tube was similarly inserted into an ear vein and advanced beyond the base of the ear. No untoward effects were observed from either manipulation.

Endogenous creatinine was determined in tungstate filtrates of plasma after adsorption on and elution from Lloyd's reagent. Urine creatinine was determined in acidified, diluted urine. In the periods in which inulin clearance was being measured, urine creatinine analysis was made after adsorption with elution from Lloyd's reagent (5).

Para-aminohippurate (PAH) and inulin were determined in cadmium sulfate filtrates of plasma and in diluted urines by the methods of Smith *et al.* (6) and Schreiner (7).

Osmolality of plasma and urine was measured by a semi-micro modification of the Johlin (8) method utilizing a thermistor and a DC bridge (9).

RESULTS

Two representative experiments in one pig are illustrated in Figure 1. This pig gained 2 kg. in weight in the interval of 5 days between experiment A and experiment B. The intravenous doses of the vasopressins, 10 mU. in each case, are equivalent to International Standard Powder as estimated from the vasopressor response of anesthetized rats. The sequence of injections was reversed in the second experiment so that three injections of each

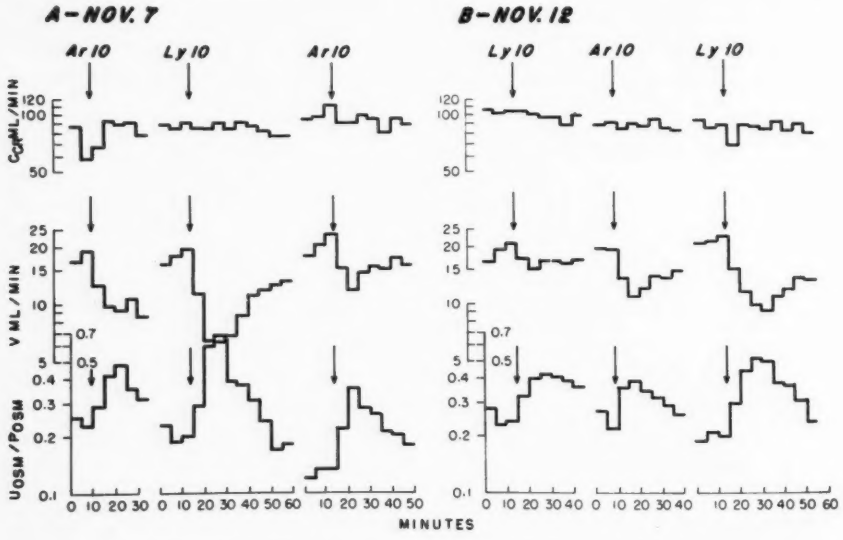

FIGURE 1

hormone could be given in the two experiments. Lysine vasopressin is the more potent hormone in two of three trials as judged by the decreased rate of excretion of urine and by the increased concentration of osmotically active substances (increase in U_{OSM}/P_{OSM} ratio). In the first injection of lysine vasopressin in experiment B, the effect on the ratio U_{OSM}/P_{OSM} is about the same as that of arginine vasopressin; on the other hand arginine vasopressin caused a greater reduction in the volume of urine in this comparison.

The clearance of endogenous creatinine is shown for both experiments. Although one large unexplained fall in clearance occurred during the first injection of experiment A and there were smaller variations later in the two experiments it is believed that these variations did not represent circulatory changes which seriously modified the renal response to the vasopressins. In four other experiments, the clearance of endogenous creatinine was similarly determined. Variations were slight and none comparable to the change during the first injection of experiment A was encountered. It will be shown later that the clearance of endogenous creatinine in the pig is not

a measure of the true rate of glomerular filtration; nevertheless it is a fairly satisfactory guide to the variations which may have occurred in experiments such as those of Figure 1.

Thirty-two comparisons of the antidiuretic activities of arginine and lysine vasopressins injected intravenously in doses of 2–10 mU. were made under conditions as nearly comparable as possible. Antidiuretic effect was expressed as percentage decrease in urine volume. In 16 of these experiments the increase in the osmolality of the urine after a vasopressin was also determined. The results are summarized in Table 1. Lysine vasopressin was the more potent in 65% of the comparisons based upon the maximum percentage decrease in the volume of urine following the injection of hormone. In the experiments in which the maximum percentage increase in the osmolality of urine was determined, lysine vasopressin was the more potent in about 80% of the comparisons. It is concluded that intravenous lysine vasopressin is as potent and possibly slightly more potent than arginine vasopressin as an antidiuretic agent in the pig.

TABLE 1

	2 mU.	3 mU.	4 mU.	5 mU.	6 mU.	8 mU.	10 mU.	Total
Percentage decrease volume								
Lys. > Arg.	6	2	5	1	2	2	4	22
Lys. = Arg.	3	—	3	2	1	—	2	11
Lys. < Arg.	—	—	—	—	—	—	1	1
Totals	9	2	8	3	3	2	7	34
Percentage increase U_{OSM}								
Lys. > Arg.	3	—	3	1	1	—	5	13
Lys. = Arg.	2	—	—	—	—	—	1	3
Lys. < Arg.	—	—	—	—	—	—	—	0
Totals	5	—	3	1	1	—	6	16

Some parameters of renal function were investigated in four experiments and the results are summarized in Table 2. The clearance of inulin is considered to represent the true rate of glomerular filtration. The clearance of endogenous creatinine was consistently lower than that of inulin. The C_{CR}/C_{IN} in 39 periods of 4 experiments on 3 pigs was 0.87 with a standard error of ± 0.015. In the experiments on pigs B and C, the clearance of both inulin and para-aminohippurate were determined. The results indicate that

TABLE 2

Pig	Body wgt., Kg.	C_{CR} (endogenous), ml./kg./min.	C_{IN}, ml./kg./min.	C_{PAH}, ml./kg./min.	Tm_{PAH}, mg./kg./min.
A	20.5	4.07 ± 0.14	4.88 ± 0.20	—	—
A	22.5	3.82 ± 0.14	5.16 ± 0.35	—	—
B	20.5	4.40 ± 0.13	5.32 ± 0.10	19.3 ± 0.54	3.21 ± 0.04
C	26.3	4.30 ± 0.11	4.87 ± 0.11	19.7 ± 0.46	3.60 ± 0.09

the filtration fraction is approximately 0.26 in the pig; the figure for man is lower (about 0.20) and that for the dog is somewhat higher (about 0.31) (10).

DISCUSSION

The experiments here reported were performed on growing dwarf pigs which proved to be excellent animals for renal function studies without anesthesia. The first interest was in the comparative effects of lysine and arginine vasopressins. Arginine vasopressin is about 6 times as potent as lysine vasopressin in inhibiting water diuresis in dogs when they are injected intravenously in equal vasopressor doses (3). When antidiuretic assays are performed by intravenous injection into rats anesthetized with ethanol, the results are difficult to evaluate because they depend upon the conditions of the experiment. For example, Thorn (11) concluded that the ratio of antidiuretic activity of arginine to lysine vasopressin is about 6 and thus resembles what was previously reported for the dog. Later experiments by Sawyer (12) led him to conclude that it is difficult to assign a definite ratio of potency of the two vasopressins after intravenous injection in rats. He concluded that the peak antiuretic effect from the two vasopressins is about the same but that arginine vasopressin has a much more persistent action.

The antidiuretic response of pigs to lysine vasopressin is of special interest because this polypeptide is the antidiuretic hormone of this mammal. Arginine vasopressin, judged from the pharmacological effects of extracts, is the secretion of all other mammals which have been investigated so far. In the experiments reported in this article lysine vasopressin was found to be as potent if not more potent than arginine vasopressin as an intravenous antidiuretic substance (Fig. 1 and Table 1). This was true whether the effect was judged from the transient decrease in the volume of urine or from the increase in the concentration of urinary solutes (U_{OSM}/P_{OSM}). This finding is in marked contrast with that in the dog. Both vasopressins produce antidiuresis in the pig, without significantly altering glomerular filtration rate.

There is very little information available concerning the physiology of the porcine kidney. Dalgaard-Mikkelsen *et al.* (13) reported inulin clearances of 3.65 ml./kg./min. in young pigs under urethane sedation. They did not measure creatinine or para-aminohippurate clearances or urine osmolality.

The experiments of this series were performed in unanesthetized pigs trained to lie in a reasonably tranquil state during the experiment. During water diuresis under a heavy water load about 20% of the glomerular filtrate is excreted and free water clearance often exceeds 18%. These proportions are unusually high for a mammal. The filtration rate of approximately 5 ml./kg./min. is also very high for a mammal of this size, being

surpassed by the rat (6 ml./kg./min.) but not by the dog, rabbit or man (4.3, 3.1 and 2.0 ml./kg./min. respectively) (10). Likewise the renal plasma flow as judged by the clearance of para-aminohippurate as well as the maximal tubular transfer of para-aminohippurate are high in the pig. In terms of body weight, C_{PAH} is higher in the rat (22 ml./kg./min. compared with 19.5 ml. in the pig) but lower in other mammals (rabbit, 18 ml.; dog, 13.5 ml.; and man, 10 ml.) (10). The maximal rate of tubular transport of para-aminohippurate was found to be about 3.1 mg./kg./min., a figure which is about 3 times as high as that for the dog and much higher than is found in man (about 1.3 mg./kg./min.) (10).

The clearance of endogenous creatinine was consistently lower than the clearance of inulin. These determinations were based on the measurement of so-called "true" creatinine adsorbed by Lloyd's reagent. It is possible that non-creatinine chromagens exist in pig plasma that were measured by this method and this could explain the low C_{CR}/C_{IN} ratio. We have not ruled out the possibility that plasma creatinine is less completely filtered than inulin by the glomeruli of the pig. These data are, therefore, only suggestive that there may be tubular reabsorption of true creatinine in the pig. If this can be substantiated by studies of exogenous creatinine clearance and the use of tubular transport blocking agents the pig may stand in sharp distinction from other well-studied vertebrates that excrete creatinine by glomerular filtration alone or by filtration and tubular secretion (10).

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DIFFERENCES IN THE ANTIDIURETIC RESPONSES OF RATS TO THE INTRAVENOUS ADMINISTRATION OF LYSINE AND ARGININE VASOPRESSINS[1]

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ABSTRACT

When injected intravenously into rats anesthetized by ethanol, lysine vasopressin causes an antidiuresis that is as intense as that caused by arginine vasopressin. Its duration is, however, less. Accurate quantitative comparison of the two vasopressins in terms of this assay for antidiuretic activity is impossible unless antidiuretic activity be rigidly defined.

ARGININE vasopressin appears to be the neurohypophysial antidiuretic hormone of cattle, man, and many other mammals. It contains arginine as the penultimate amino acid in the polypeptide chain. Hog neurohypophysial vasopressin, however, contains lysine in this position. Commercial vasopressin is derived from either hog or cattle pituitaries or from mixtures of both. Pharmacologically the two vasopressins differ significantly. van Dyke, Engel and Adamsons (1) have found that one pressor unit of lysine vasopressin has but one sixth of the antidiuretic activity of a similar quantity of arginine vasopressin when assayed by intravenous injection in dogs. If arginine vasopressin, therefore, is defined as having an antidiuretic to vasopressor potency ratio of 1.00 then this ratio for lysine vasopressin in the dog is only 0.16. If lysine vasopressin is assayed for antidiuretic activity by subcutaneous injection in rats, however, its antidiuretic to vasopressor ratio is approximately 1.00.

The difference in antidiuretic potency between lysine and arginine vasopressins as assayed in rats and dogs could result from a species difference or be due to the different routes of administration. We will demonstrate that a species difference does certainly exist and that there are qualitative differences in the antidiuretic effects of these hormones in the rat that are such that quantitative comparisons can be only approximate.

METHODS

We have used a modification of the Jeffers, Livezy and Austin (2) assay for antidiuretic activity by intravenous injection in rats anesthetized with ethanol. The water

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load is kept constant as recommended by Dicker (3) at 8–10% of the body weight. This level of hydration is maintained by the injection of 1.5% ethanol in 0.05% NaCl by stomach tube at rates sufficient to maintain a constant weight. This is simpler than rehydrating with a volume equal to the urine excreted plus an arbitrary excess to compensate for extrarenal water loss. The maintenance of a constant weight has the additional advantage that it can be made automatic with a much simpler apparatus than the outflow-meter relay-controlled device of Boura and Dicker (4). Two stainless steel wires, one connected to the beam of a balance, are arranged to make contact and activate a relay whenever the weight of the rat decreases by 0.2 gm. The relay drives a pump that administers the hydrating solution to the rat until its weight has returned to the selected value and the contact is broken.

Urine flow is recorded as drops each minute by a Thorp impulse counter.

Injections are given in a constant total volume including wash (usually 0.2 ml.) through a polyethylene cannula threaded through the external jugular vein into the superior vena cava. This assures that the administered dose enters the circulation by way of an open and rapidly flowing vein. An interval of at least 50 minutes is allowed to elapse between injections. A balanced four point assay design is usually employed, each rat receiving at least two doses of standard and two doses of unknown. Potency ratio for each assay is calculated on the assumption that the slopes of the regressions of response on log dose for unknown and standard are identical. This identity for lysine and arginine vasopressins is assumed for convenience of calculation but the validity of this assumption has not been established.

Vasopressor activity is determined by intravenous injection in rats (5).

In some of the experiments a conductivity cell made of two short lengths of stainless steel needle tubing separated by about 2 mm. of polyethylene tubing was inserted in the catheter leading from the bladder. Another length of needle tubing was inserted in the catheter between the rat and the conductivity cell. This tubing was passed through a loop of Tygon tubing through which water at 25° C was actively circulated. This arrangement was adequate to cool the urine to approximate room temperature prior to conductivity measurement. The entire dead space from kidney to conductivity cell was estimated to be less than 0.1 ml.

RESULTS

We have previously used a certain lot of Parke, Davis Pitressin (Lot No. M370B) as a working standard for pressor and antidiuretic assays. When we recently restandardized this lot we took the precaution of determining its antidiuretic activity by intravenous assay in rats as well as its vasopressor activity. A significant discrepancy appeared between the vasopressor and antidiuretic assays as compared to the reference standard. The antidiuretic activity was only about 76% of the vasopressor activity.

van Dyke, Engel and Adamsons (1) reported that lysine vasopressin has an antidiuretic to vasopressor ratio of about 0.16 in the dog and Thorn (6) reported a similar ratio based on intravenous administration in rats. We thought, therefore, that the Pitressin sample contained a mixture of lysine and arginine vasopressins, and that this accounted for its intermediate ratio of 0.76. The manufacturer, however, informed us that this lot of Pitressin was derived wholly from hog pituitaries. It contained, therefore, only lysine vasopressin. The high antidiuretic to vasopressor ratio was in apparent

TABLE 1. ANTIDIURETIC TO VASOPRESSOR RATIOS OF ARGININE AND LYSINE VASOPRESSINS BASED ON INTRAVENOUS INJECTION IN RATS

Preparation	Number of assays	Measure of "antidiuresis"	
		10 minute average urine flow	Minimum urine flow
Arginine vasopressin	—	1.00	1.00
Pitressin M370B (hog)	6	0.76 ± 0.086	0.90 ± 0.135
Lysine vasopressin	10	0.73 ± 0.090	0.92 ± 0.106

conflict with the findings of Thorn who had used an assay basically similar to ours.

We proceeded, therefore, to repeat the comparison of antidiuretic and vasopressor activities of the vasopressins using highly purified natural lysine and arginine vasopressin prepared in Dr. V. du Vigneaud's laboratory.

By our standard intravenous assay technique in the rat purifed lysine vasopressin appears to have an antidiuretic to vasopressor ratio of 0.73 (Table 1). The measure of antidiuresis in this method is the ratio of urine flow for 10 minutes following injection to the urine flow for 10 minutes prior to injection. Since this result is again in striking conflict with Thorn's (6) report we decided to try to approximate his assay more closely. He measured antidiuresis in terms of the increase in specific gravity of the urine collected during the 20 minute period following injection. We felt

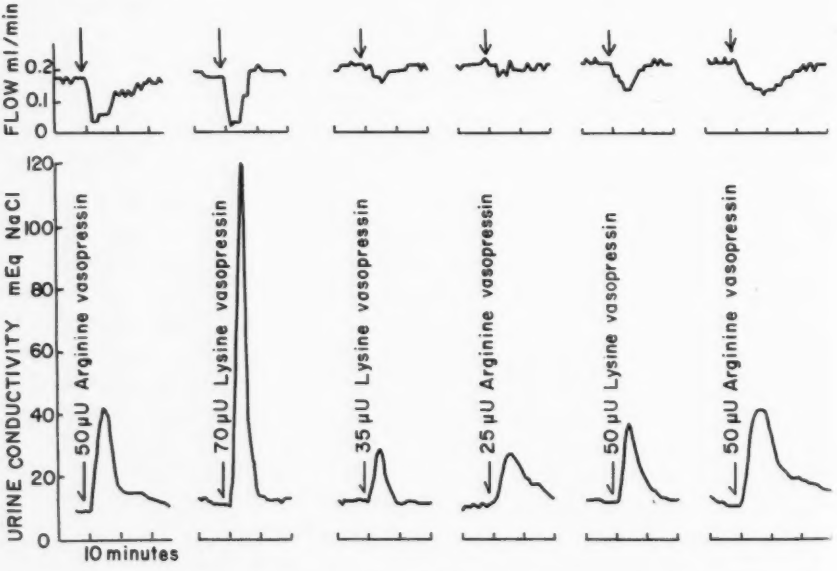

FIG. 1. Antidiuretic responses of a 228 gm. rat to the intravenous injection of lysine and arginine vasopressins at several dose levels.

that specific gravity was a doubtful measure of osmolar concentration, particularly in the presence of high urinary ethanol concentration. Ethanol also makes freezing point or vapor pressure determinations difficult to interpret. We therefore chose to follow conductivity changes as a rough estimate of osmolar concentration. Under these conditions the bulk of urinary solute is present as NaCl. Conductivity has the additional advantage of being suitable for continuous monitoring and automatic recording.

The plotting of conductivity following arginine and lysine vasopressins (Fig. 1) reveals a very characteristic difference. The antidiuresis after lysine vasopressin is much briefer than that following arginine vasopressin. The same difference is evident in the plot of urine flow but was not appreciated until after conductivity changes had been observed. As a consequence of the difference in the time course of antidiuresis after lysine and arginine vasopressins any precise quantitative comparison becomes impossible. If a long collection period is measured (Thorn (6) used 20 minute periods and administered vasopressins in doses approximately 10 times as great as we find necessary) arginine vasopressin appears more potent. If, on the other hand, the intensity of antidiuresis irrespective of duration is the criterion of activity the difference between arginine and lysine vasopressins becomes insignificant. If antidiuresis is measured as the maximum depression of urine flow (in practice, the minimum urine flow over a three-minute period) the antidiuretic to vasopressor ratio of lysine vasopressin approaches 1.0 (Table 1). The discrepancy between the duration of antidiuresis after high doses of lysine and arginine vasopressins is also well illustrated in the urine volume plot in the third figure of Thorn's paper (6) but he does not comment on this.

Conductivity changes were inversely proportional to the changes in urine flow. Estimations of antidiuretic activity based on conductivity changes were essentially the same as those based on minimum urine flow. The scatter of the data was greater, probably as a result of unavoidable mixing of urine in the dead spaces between kidneys and conductivity cell. Concentration changes in this preparation, therefore, as estimated by conductivity measurement, do not appear to offer a useful basis for quantitative assay of antidiuretic activity.

DISCUSSION

Thorn (7) expresses the belief that his findings in rats (6) and van Dyke, Engel and Adamsons' (1) observations in dogs indicate that the low antidiuretic to vasopressor potency ratio of lysine vasopressin is an inherent property of the molecule and that these results constitute proof that the difference in relative antidiuretic potency of arginine and lysine vasopressins is not dependent upon species. The observations reported in this paper, that lysine vasopressin has approximately as intense an antidiuretic effect

as arginine vasopressin when injected intravenously into rats, clearly indicate that a species difference *does* exist between the rat and the dog. In the latter, intravenous lysine vasopressin exerts a much weaker antidiuretic effect in terms of both intensity and duration than does arginine vasopressin. The importance of species variation in response is further illustrated by the report of Munsick, Sawyer and van Dyke (8) that lysine vasopressin is as potent or more potent as an antidiuretic agent than is arginine vasopressin when administered intravenously to pigs.

Although intravenous lysine vasopressin produces as *intense* an antidiuresis in the rat as arginine vasopressin the *duration* of antidiuresis is less. This qualitative difference makes quantitative estimation of relative potencies dependent upon the definition of antidiuresis that one chooses and upon the duration of urine collection periods that one records as a basis for the measure of antidiuresis.

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NOTES AND COMMENTS

NATURE OF RADIOIODINE NOT DISCHARGEABLE WITH PERCHLORATE IN THE THYROID GLANDS OF THIOURACIL-TREATED RATS

ABSTRACT

A chromatographic investigation of the thyroidal I^{131} which is non-dischargeable by sodium perchlorate in thiouracil-propylthiouracil treated rats has been made.

At least part of the non-dischargeable I^{131} has been shown to be bound to protein.

Tryptic hydrolysis of this protein shows that it contains organic I^{131} as monoiodotyrosine only.

Halmi and Stuelke (1) have recently shown that a dose of sodium perchlorate ($NaClO_4$) which completely inhibits active collection of I^{131} by the thyroid glands of propylthiouracil (PTU) treated rats, fails to discharge all the accumulated I^{131} when given after I^{131} administration. Gross (cf. 2) has observed that part of this I^{131} not discharged by $NaClO_4$ remains at the origin during the chromatographic analysis and is presumably protein-bound. It was thought of interest to investigate the nature of this I^{131}-containing fraction.

METHODS AND MATERIALS

Adult male hooded rats weighing between 150 and 200 gm. were maintained on the Bruce and Parkes diet 41 (3), and most of them were given 0.1% thiouracil (TU) in 5% sucrose solution to drink, *ad libitum*. The animals were kept on this regimen for 10 days to 4 weeks before the experiments with $NaClO_4$ or other anions that inhibit thyroidal iodide collection. $NaClO_4$ or the other salts were injected intraperitoneally, under light ether anesthesia. Carrier free I^{131} was injected subcutaneously in physiological saline solution. The experiments were performed as shown in Table 1.

TABLE 1. TREATMENT SCHEDULES

No. of rats	Pre-treatment	First injection	Time after first injection (min.)		
			60	120	180
12	TU	5 mg. PTU	200 μC. I^{131}	100 mg. $NaClO_4$	kill
2	TU	5 mg. PTU	200 μC. I^{131}	10 mg. $NaClO_4$	kill
2	—	5 mg. PTU	200 μC. I^{131}	100 mg. $NaClO_4$	kill
4	TU	5 mg. PTU	20 μC. I^{131}	—	kill
1	—	5 mg. PTU	200 μC. I^{131}	—	kill
2	—	100 mg. $NaClO_4$	1000 μC. I^{131}	kill	
1	TU	100 mg. $NaClO_4$	200 μC. I^{131}	kill	
1	—	50 mg. $NaClO_4$	500 μC. I^{131}	50 mg. $NaClO_4$	kill
3	TU	5 mg. PTU	200 μC. I^{131}	10, 25 or 100 mg. Na SCN	kill
2	TU	5 mg. PTU	200 μC. I^{131}	100 mg. $NaClO_3$	kill
2	TU	5 mg. PTU	200 μC. I^{131}	100 mg. $NaNO_3$	kill
2	—	20 μC. I^{131}	—	kill	

The rats were killed by exsanguination under ether anesthesia, the thyroids were removed, and subjected to the following treatments:

1. A small piece of each lobe was snipped with scissors and pressed with a flattened glass rod onto Whatman filter paper No. 1 at the point marked origin (O), and allowed to dry.
2. Portions of each lobe were heated in a water bath at 80–85° in 0.5N NaOH for 10–15 minutes.
3. Portions of each lobe were hydrolysed with trypsin in NH_3-NH_4Cl-urea or borate

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buffers at pH 8.5 for 24 hours. Aliquots of the hydrolysates and the unhydrolysed thyroid were analysed by ascending chromatography in *n*-butanol saturated with 2N-acetic acid (BAc) and in *n*-butanol-dioxan-2N-NH_3 (4:1:5)(BDA). TU was added to both solvents to prevent oxidation of iodide on the chromatograms. Monoiodotyrosine (MIT), diiodotyrosine (DIT), thyroxine (T_4) and iodide were used as carriers to identify the radioactive compounds present. The iodo-amino acids were stained by the method of Gross and Leblond (4); iodide was stained with palladium chloride. The radioactivity on the chromatograms was measured with an automatic strip counter.

Saline extracts of the thyroid glands from normal and PTU-$NaClO_4$ treated rats were compared by paper electrophoresis in a buffer containing 0.5*M* TRIS, 0.021*M* versene and 0.075*M* boric acid, and in two barbital buffers: sodium barbital-acetic acid-KCl, pH 7.5 and sodium barbital-HCl, pH 8.6.

RESULTS

The results are shown in Figures 1, 2 and 3. Figure 1A shows that in the thyroid gland of normal rats the I^{131} was contained predominantly in thyroglobulin 2 hours after injection—this remained at the origin in BDA (and in BAc). Figure 1B shows that this thyroglobulin, after 24 hours' tryptic digestion, was found to contain mostly MIT and DIT. (We have found that in our rats labeled T_4 is not always present in the thyroid 2 hours after I^{131} injection.) Figure 2C demonstrates that rat thyroid after PTU alone followed by I^{131} contained only iodide. This result was obtained both with previously untreated animals and with animals pretreated with TU. After PTU and I^{131} followed by $NaClO_4$ (Fig. 2D) the thyroid gland contained iodide together with organically bound iodine which remained, like thyroglobulin, at the origin of the chromatogram. Tryptic hydrolysates of the glands, when chromatographed in BDA (Fig. 2E) and BAc (Fig. 2F) showed that the bound organic iodine was in the form of MIT only. The amounts of protein-bound-MIT (PB-MIT) obtained were variable but sometimes were as high as 60% of the total I^{131} in the gland. In the thyroid of a rat which was given TU chronically and $NaClO_4$ before the injection of I^{131} radioactivity was predominantly in the form of iodide, but some did remain at the origin. There was not enough activity in the latter fraction to permit chromatography after hydrolysis.

When rats were treated with $NaClO_4$ without preceding TU or PTU administration, only diffusion of I^{131} presumably took place. Figure 3G shows that this iodine was organically bound in the thyroid; after hydrolysis a "normal" (i.e. thyroglobulin-like) distribution of MIT, DIT and T_4 was observed (Fig. 3H).

It appears from these experiments that at least part of the non-dischargeable iodine in thyroids of rats treated with PTU before and with $NaClO_4$ after I^{131} administration

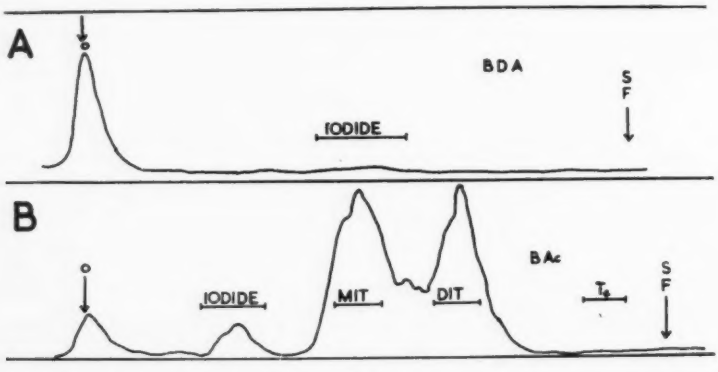

FIG. 1. Chromatographic analysis of I^{131}-labeled thyroglobulin in normal rat thyroid A: before hydrolysis; B: after tryptic hydrolysis. In this and the subsequent figures O denotes origin, SF solvent front.

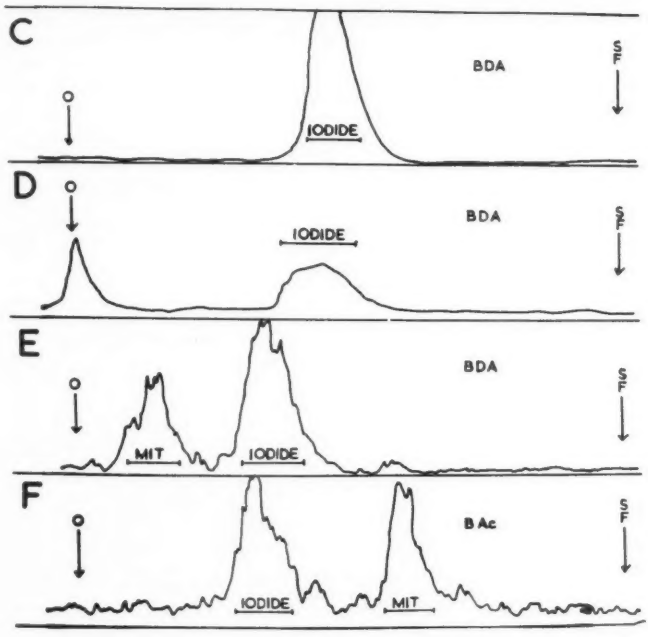

FIG. 2. C. Chromatographic analysis of thyroid from rat treated with PTU followed by I^{131}. D. Chromatographic analysis of thyroid from rat treated with PTU, I^{131} and $NaClO_4$. E, F. Same as D, after tryptic hydrolysis.

consists of a protein containing I^{131} as MIT only. Even when animals were killed 5 hours after I^{131} administration, with hourly injections of 20 mg. of $NaClO_4$ starting 1 hour after I^{131} (not shown in Table 1), labeled MIT alone was found in the protein hydrolysate.

The PB-MIT was electrophoretically indistinguishable from normal thyroglobulin. This contrasts with the results of Gross (cf. 2) who found that unlike normal thyroglobu-

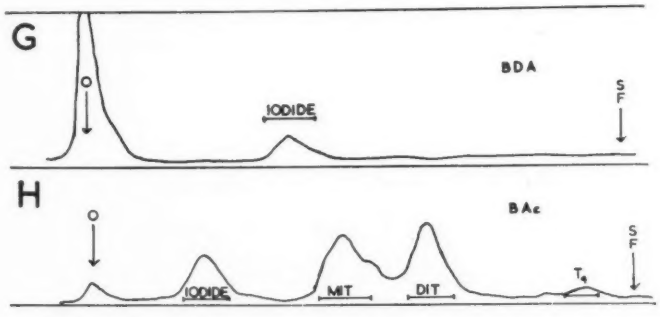

FIG. 3. Chromatographic analysis of I^{131}-labeled thyroglobulin in thyroid of rat which received no TU or PTU, and I^{131} following $NaClO_4$ administration. G: before hydrolysis; H: after tryptic hydrolysis.

lin this non-dischargeable I^{131} possessed little electrophoretic mobility in barbital buffer at pH 8.6; the reason for this discrepancy is unknown.

The PB-MIT was sometimes found to be more labile to alkaline hydrolysis than was normal thyroglobulin. In some experiments all the MIT was liberated by heating for 10 minutes at 85° in 0.5N NaOH. Similar treatment of normal rat thyroglobulin gave rise to compounds with a low chromatographic mobility which were presumably MIT and DIT peptides, since further hydrolysis liberated both these amino acids.

PB-MIT was also formed when smaller doses of $NAClO_4$ (10 mg.), sodium thiocyanate (NaSCN), sodium chlorate ($NaClO_3$) or sodium nitrate ($NaNO_3$) were administered; with the three last-named compounds (see Table 1 for dosages) the amounts formed were very small, rarely exceeding 5% of the total I^{131} in the gland.

COMMENT

TU and PTU treatment completely inhibit iodine binding in the thyroid gland, but permit iodide collection to proceed. However, $NaClO_4$ given to rats pretreated with TU and/or PTU does not discharge all the concentrated iodide, and in fact permits a small amount of organic incorporation to take place. If, as has been suggested, the thiol-antithyroid drugs act partly by keeping iodide in the reduced state, $NaClO_4$ might, in the high concentrations used in these experiments, interfere with the iodide-thiouracil reaction.

This, however, is not likely to be the complete explanation. The only iodination process which is restored by $NaClO_4$ to the PTU-blocked gland is that of tyrosine to MIT; no further iodination to DIT takes place. In this connection it is interesting to note that in hydrolysates of certain non-toxic nodular goiters (5) and thyroid cancers (6) the proportion of MIT to other iodo-amino acids is very high. Further, thyroid homogenates can bind iodine in organic linkage, but only as MIT (7), as can early fetal thyroids before they acquire the ability to produce thyroid hormone (8). Lastly the mammary gland (9, 10) also incorporates iodine into protein linkage and secretes the iodoprotein into milk: this protein on hydrolysis also yields predominantly MIT. It appears therefore that the organic binding of iodine to MIT is a primitive process which can take place in other organs and in disorganized, incompletely developed or disrupted thyroid tissue. Further elaboration to the thyroid hormones must require special enzymes which are only present in the thyroid and which depend for their activity on the integrity of the cell.

The organic binding of iodine which takes place in thiocarbamide-blocked thyroids after the administration of $NaClO_4$ (and probably owing to the action of this ion) resembles the primitive process of bound MIT formation seen in the conditions listed above, rather than the process of thyroid hormone biosynthesis.

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THE ENDOCRINE SOCIETY

1959 Annual Meeting

The Forty-First Annual Meeting of The Endocrine Society will be held in the Haddon Hall Hotel, Atlantic City, New Jersey, Thursday, Friday, and Saturday, June 4, 5, 6, 1959.

The Chairman of Local Arrangements is Dr. Matthew Molitch.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 5th at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Chalfonte-Haddon Hall Hotel will hold 300 bedrooms for members until May 1, 1959, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. Make your reservations now and avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 1st 1959 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Frank Engel, Duke Hospital, Durham, North Carolina, not later than February 1, 1959. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. The Council requests that authors adhere strictly to the following rules when submitting abstracts, otherwise they cannot be considered:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.

2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.

3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.

Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.

Line 3. Institution of origin and city in which institution is located.

4. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.

5. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1958.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by October 15, 1958.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

NATIONAL ACADEMY OF SCIENCES

The Division of Medical Sciences of the National Academy of Sciences—National Research Council is accepting applications for grants-in-aid of research for consideration by the Committee for Research in Problems of Sex. The funds for support of this program are provided by the Rockefeller Foundation and the Ford Foundation. The Committee is concerned primarily with encouraging research on the mechanisms underlying sexual behavior, with special emphasis on the higher mammals and man. Proposals involving endocrinological, neurological, psychological, anthropological, phylogenetic, and genetic studies directed toward this objective are therefore invited. Requests that deal with the physiology of reproduction or with related biological and biochemical fields should be addressed to the Committee only if they give promise of shedding light upon behavioral mechanisms.

Preliminary inquiries should be addressed to Room 411, Division of Medical Sciences, National Academy of Sciences—National Research Council, 2101 Constitution Avenue, N. W., Washington 25, D. C. Completed applications for the fiscal year 1959–1960 should be postmarked on or before January 15, 1959.

CORRECTION

In the article by Taurog, Tong and Chaikoff "Thyroid I^{131} Metabolism in the Absence of the Pituitary" which appeared in Endocrinology *62*, 646 (May) 1958, the value in Table 1, line 2, column 3, should be 0.039, instead of 0.39.